Early events in dengue virus infection

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PhD Thesis

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Part I

General introduction and scope of the thesis

Chapter 1

Recent Development in Understanding Dengue Virus Replication

Silvio Urcuqui-Inchima, Claudia Patino, Silvia Torres, Anne-Lise Haenni, and Francisco Javier Dıaz

Dengue is the most important cause of mosquito-borne virus diseases in tropical and subtropical regions in the world. Severe clinical outcomes such as dengue haemorrhagic fever and dengue shock syndrome are potentially fatal. The epidemiology of dengue has undergone profound changes in recent years, due to several factors such as expansion of the geographical distribution of the insect vector, increase in traveling, and demographic pressure. As a consequence, the incidence of dengue has increased dramatically. Since mosquito control has not been successful and since no vaccine or antiviral treatment is available, new approaches to this problem are needed. Consequently, an in-depth understanding ofthe molecular and cellular biology of the virus should be helpful to design efficient strategies for the control of dengue. Here, we review the recently acquired knowledge on the molecular and cell biology of the dengue virus life cycle based on newly developed molecular biology technologies.

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Abbreviations

aa, amino acid; ADE, antibody-dependent enhancement; C, capsid / coat protein; Cdc42, cell division cycle 42; cHP, capsid hairpin; CS, cyclization sequence; DAR, downstream of the AUG region; DC, dendritic cells; DC-SING, dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin; DENV, dengue virus; DF, dengue fever; DHF, dengue hemorrhagic fever; ds, double-stranded; DSS, dengue shock syndrome; E, envelope; eEF, eukaryotic elongation factor; eIF, eukaryotic initiation factor; ER, endoplasmic reticulum; FcgR, receptor of the Fc portion of IgG; GFP, green fluorescent protein; HLA, histocompatibility-linked antigen; HMEC, human microvascular endothelial cell; HSP, heat shock protein; IF, intermediate filament; IFN, interferon; IL, interleukin; LC3-II, light chain 3 form II of the microtubule-associated protein 1; LD, lipid droplet; M, matrix protein; MT, microtubule; MTase, methyltransferase; NC, nucleocapsid; NS, non-structural; nt, nucleotide; NTPase, nucleoside triphosphatase; ORF, open reading frame; PABP, poly(A)-binding protein; prM, precursor of M; Rab 5, Rabatin-5; Rac1, ras-related c3 botulinum toxin substrate 1; RC, replication complex; RER, rough ER; RdRp, RNA-dependent RNA polymerase; RTPase, RNA 5'-triphosphatase; siRNA, small interfering RNA; SL, stem-loop; sgRNA, subgenomic RNA; ss single-stranded; TGN, trans-Golgi network; TNF, tumor necrosis factor; UAR, upstream AUG region; UTR, untranslated region; V-ATPase, vacuolar ATPase; WHO, World Health Organization.

I. INTRODUCTION

Although dengue only became a major global medical concern within the last few decades, disease that might have been caused by dengue was reported in China as early as the VIIth century. Since then, epidemics that may have been caused by dengue have sporadically appeared with outbreaks in the XVIIth century in Central America and in Philadelphia in the XVIIIth century. In the XXth century epidemics were reported in Southeast Asia and the Pacific, favored by increased expansion of the dengue virus (DENV) mosquito vector, *Aedes aegypti*. This was accompanied by circulation of the four DENV serotypes (known as hyperendemicity) and the appearance of dengue hemorrhagic fever (DHF). By the 1970's, DHF had become a major cause of death among children in regions where DHF occurred. Since then, epidemics have become more frequent and more intense, and cover an ever increasing geographical area. Although before the 1980's Africa had not experienced major epidemics, it now harbors all four DENV serotypes (reviewed in Gubler, 1998). DENV continues to be a public health problem in tropical and subtropical countries. With the recent increase in dengue epidemics, interest in attempts to control dengue has expanded. It has become urgent to control dengue because to date neither animal model, vaccine, nor therapeutic measures are available to counteract DENV and the disease. One of the control measures adopted had been vector control. However, although this strategy seemed to be highly promising, it was later neglected, resulting in renewed epidemics in various parts of the world. Safe and efficient strategies will need to be developed to control the vector, and will require the active participation of local communities. The aim of the present review is to bring together recent knowledge acquired on the molecular and cell biology of the life cycle of DENV in the hope that it might lead to a better understanding of dengue diseases. References to recent review articles related to the field of research presented here can be found throughout the present review.

II. DENGUE DISEASE

A. The agent

Dengue is the most widespread arthropod-borne viral disease of humans and is caused by DENV. DENV is a spherical, enveloped virus with a monopartite single-stranded (ss) positive-sense RNA [ssRNA(+)] of the family *Flaviviridae*, genus *Flavivirus* (reviewed in *Mukhopadhway et al., 2005*). Four DENV

serotypes are recognized, DENV-1 to DENV-4 that possess 67-73% identity at the nucleotide (nt) level and 69-78% identity at the amino acid (aa) level. Three to six subtypes (genotypes) can be distinguished within each serotype (Diaz *et al.,* 2006). Monoclonal antibodies made it possible to identify epitopes that are serotype-specific, DENV complex-specific and flavivirus group-reactive epitopes as well as intermediate categories. All serotypes have the same transmission cycles and cause similar clinical manifestations although serotype- and strain-related differences in virulence exist. Infection by one serotype is believed to confer life-long immunity to only that serotype (reviewed in Rico-Hesse, 2010).

B. Eco-epidemiology

DENV is vectored by *Aedes* mosquitoes. Humans and *A. aegypti* are generally the only vertebrate reservoir and vector respectively, but forest and rural cycles involving nonhuman primates and *Aedes* species other than *A. aegypti* exist. *A. aegypti* breeds mainly in human-made containers and about 2.5 billion people are at risk in regions where *A. aegypti* is endemic (www.who.int/mediacentre/factsheets/fs117/en/). A general tendency towards increased frequency and magnitude of dengue outbreaks has occurred recently. The reasons invoked are demographic, cultural, environmental and political (Guha-Sapir and Schimmeer, 2005; Wilder-Smith and Gubler, 2008). Prominent factors implicated are increase in global population and in air travel, and rapid and unplanned urbanization. This has resulted in the simultaneous circulation of multiple DENV serotypes, known as hyperendemicity. Other factors include the increasing use of disposable products that become mosquito breeding sites, decay in public health infrastructure, insecticide resistance, and global warming.

C. Clinical manifestations

Infection by DENV results in clinical outcomes ranging from asymptomatic to fatal. Traditionally, dengue cases have been classified as undifferentiated fever, dengue fever (DF) and DHF; DHF has been further divided in cases with or without shock, the first being designated as dengue shock syndrome (DSS). A more recent classification divides the disease into dengue (with or without warning signs) and severe dengue; the second category includes cases with significant plasma leakage, hemorrhage or organ impairment, comprising the old categories of DHF and DSS, as well as other severe forms of DENV-induced disease that affect the brain, liver, heart or other organs [World Health Organization (WHO), 2009]. Although this latter classification promises improved reporting of cases and better medical management, here we will often use the more familiar DF, DHF and DSS designations.

At least half of the DENV infections are asymptomatic or mild. Yet DF is an acute disease characterized by fever and pains in the head, eyes, muscles and joints. Lymphadenopathy, rash, nausea and minor hemorrhages also occur. The course is usually benign and self-limited. A variable proportion of cases evolves into DHF, a condition defined by fever, thrombocytopenia (<100,000 platelets/mm^3), hemorrhages (or a positive tourniquet test) and increased vascular permeability. DHF usually appears three to eight days after fever begins. It is often preceded by acute abdominal pain and is commonly characterized by hepatomegaly and bleeding from the skin, mucosae and the gastrointestinal tract. Most severe DHF cases develop hypotension and other signs of shock that characterize DSS, and can lead to death unless prompt fluid replacement therapy is administered (reviewed in Gubler, 1998; Guzman and Kouri, 2002).

D. Pathogenesis

No appropriate animal models exist to study DENV infections. Mice can be infected intravenously but neither viremia nor disease develops. Monkeys develop an infection but viremias are low and no disease is observed. Thus, human infections have supplied most of the information available.

Human infection begins in the skin with the injection of DENV by the bite of an infected mosquito. DENV probably initially replicates in dendritic cells (DC) or macrophages (Wu *et al.*, 2000; Markovich, 2001) and then migrates to regional lymph nodes from where it spreads to other lymphoid organs through the blood stream. It is detected in the plasma and in peripheral blood mononuclear cells. Monocytes, macrophages and DCs are major targets of DENV replication, although Kupffer cells, hepatocytes and lymphocytes have also been implicated as target cells (Halstead *et al.*, 1977; King *et al.*, 1999). Antibody response to DENV infection is different in the first (primary) and subsequent (secondary) infections. In the former, a strong IgM response appears by the end of the febrile period, and lasts two to three months; IgG appears shortly after IgM, increases moderately and is maintained for years. These antibodies are predominant against the infecting serotype but a low titer cross-reactive (heterotypic) response is also observed. After a subsequent infection, IgG levels increase more rapidly and to higher levels than in primary infections; IgM response is weaker and declines rapidly; antibodies are mostly heterotypic and titers are usually higher against the first infecting serotype (Vaughn *et al.*, 1997). Antibodies recognize both structural and non-structural DENV proteins, but only those reactive with the E (envelope), M (matrix) and non structural (NS) 1 proteins have neutralizing properties (reviewed in Pierson and Diamond, 2008). The duration of protection against re-infection with DENV remains unclear. Humans challenged with the homologous strain were completely immune to re-infection for as long as 18 months. Temporal cross-protection to challenge with a heterologous serotype was demonstrated two to nine months after the primary infection. DENV also elicits specific $CD4^+$ and $CD8^+$ T cell immune responses. After primary infection, most circulating T lymphocytes are serotype specific, but following a second infection with a different serotype, cross-reactive memory T cells predominate and more frequently recognize highly conserved proteins, especially NS3. Both $CD4^+$ and $CD8^+$ T lymphocytes can lyse DENV-infected cells. DENV-specific lymphocytes release mostly Th1 cytokines, which could be important factors in viral clearance as well as in the pathogenesis of DHF (reviewed in Mathew and Rothman, 2008).Features of DHF/DSS are increased vascular permeability, bleeding and hepatic compromise. Fatal cases present bleeding in the viscera and mucosal membranes, liquid collection in serous cavities and midzonal necrosis in the liver. Although mostly a vascular illness, vascular endothelial damage is not observed in DHF, suggesting a functional rather than anatomical alteration in the endothelium (reviewed in Gubler, 1998; Guzman and Kouri, 2002; Trung and Wills, 2010). Severe forms of DHF/DSS are associated with young age, female gender, non-African ancestry and pre-existence of immunity against other serotypes. Most severe cases are associated with a heterotypic antibody response confirming secondary infection as the main risk factor for DHF/DSS (Sangkawibha *et al.*, 1984; Burke *et al.*, 1988; Kliks *et al.*, 1989). The finding of pre-existing heterotypic antibodies in cases of DHF is linked to a phenomenon designated antibody-dependent enhancement (ADE). It consists in increased replication of certain viruses in cell culture by the addition of immune sera at dilutions beyond the neutralization endpoint. This led to the "immune enhancement hypothesis", whereby non-neutralizing antibodies remaining after the first dengue episode bind to the new infecting serotype enhancing virion penetration into monocytes and macrophages by interacting with the receptor for the Fc portion of IgG, known as FcγR (see Section IV. Cell Cycle of DENV. A. Entry and fusion complex formation: E protein and cell receptors). This phenomenon would lead to enhanced viral replication and release of soluble factors that would mediate increased vascular permeability and hemostatic disorder (Halstead and O'Rourke, 1977; reviewed in Halstead, 1981). In spite of considerable efforts, the mechanism involved in ADE remains unclear. Dejnirattisai et al. (2010) using monoclonal antibodies against the precursor of M (prM) showed that these antibodies even though they cross-react with the various DENV serotypes, are unable to block DENV infection, but on the contrary promote ADE. This interaction is likely due to some uncleaved prM

remaining on the virus surface of extracellular virions that would allow their recognition by the monoclonal antibody. Cellular immune factors are also involved: DENV-specific $CD4^+$ cells release cytokines such as interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α and TNF-β (Gagnon *et al.,* 2002). TNF-α and IL-2 can induce plasma leakage, and IFN-γ enhances TNF-α production by monocytes. IFN-γ also upregulates the expression of class II histocompatibility-linked antigens (HLAs) and of the receptor FcγR (the Fc portion of IgG) in monocytes; this could enhance DENV antigen presentation and antibody-mediated uptake of the virus, respectively. Increased levels of TNF-α, IL-6, IL-8, IL-10, and of soluble IL-2 and TNF receptors occur in DHF (Hober *et al.,* 1993; Chang and Shaio, 1994). Immunity against a different serotype is neither necessary nor sufficient for DHF/DSS development. Severe forms of DENV can occur in primary infections (Barnes and Rosen, 1974; Morens *et al.,* 1987), whereas in other settings DHF/DSS was never observed in spite of frequent sequential infections by different serotypes. Viral risk factors associated with severe forms of DENV-induced disease include the infecting serotype, subtype and strain, the time lapse between infections and the order of the serotypes in successive infections. A compelling demonstration of the importance of the virus strain in the severity of dengue came from the Americas where epidemic DHF emerged abruptly in 1981 when a Southeast Asian subtype of DENV-2 was introduced. This subtype then spread across the Caribbean, Central and South America displacing the prevalent "American subtype" of DENV-2, which had been associated with mild DF cases only (Gubler and Clark, 1995; Watts *et al.,* 1999). The time of arrival of the Southeast Asian subtype coincided with the emergence of DHF/DSS in each of these regions. At the phenotypic level, certain DENV isolates are more infectious and are disseminated more efficiently by *A. aegypti* mosquitoes than other isolates (Gubler *et al.,* 1978). At the genotypic level, consistent differences between isolates of two DENV-2 subtypes were detected in several viral genes involved in severity (Cologna and Rico-Hesse, 2003). Thus even minor differences at the molecular level can affect the clinical outcome. Yet the specific mechanisms involved are unresolved because the role of most viral proteins remains elusive. Elucidation of these roles is of utmost importance to understand the molecular basis of virulence and attenuation, which is fundamental to the design of the long-awaited safe and efficacious vaccines for dengue. This review will now focus on the molecular aspects of DENV replication to describe the viral and cell factors that could be modulating the clinical and epidemiological behavior of dengue.

III. DENV GENOME AND DENV PROTEINS

The ssRNA(+) of DENV (Figure 1B) acts directly as mRNA for the synthesis of the viral polyprotein (Figure 1C). The genome is approximately 11 kb, bears a type I cap structure (m^7GpppAmG) at its 5' end, but lacks a 3' poly (A) tail. It encodes a single open reading frame (ORF)flanked by highly structured 5′ and 3′ untranslated regions (UTRs) of about 100 and 400 nts respectively (Figure 1B); these regions are important for translation regulation, viral RNA replication (Figure 1A) and severity of infection (Leitmeyer *et al.,* 1999; Edgil *et al.,* 2003; Holden and Harris, 2004; reviewed in Markoff, 2003; Bartenschlager and Miller, 2008). The cap structure is added co-transcriptionally by the virus-encoded NS5 protein which contains in a single domain both (guanine-N7)- and (adenosine-2'-O)-methyltransferase (MTase) activities, and an as yet unidentified guanylyltransferase domain (Egloff et al., 2007; reviewed in Davidson, 2009). The RNA 5'-triphosphatase (RTPase) domain of NS3 is also involved in capping the viral RNA; although the precise role of NS3 in this process has not been well characterized, several studies have shown that the NS3 RTPase and the NS5 MTase work together removing the terminal γ-β phosphate and performing sequential guanine-N7 and adenosine-2'O methylation, respectively (Wengler and Wengler, 1993; Egloff *et al.,* 2002). The cap structure presumably stabilizes the mRNA and allows efficient translation (reviewed in Furuichi and Shatkin, 2000). The ORF is translated as a single polyprotein of 3,387-3,392 aa that undergoes co- and post-translational cleavages by viral and host cell proteinases (Figure 1C).

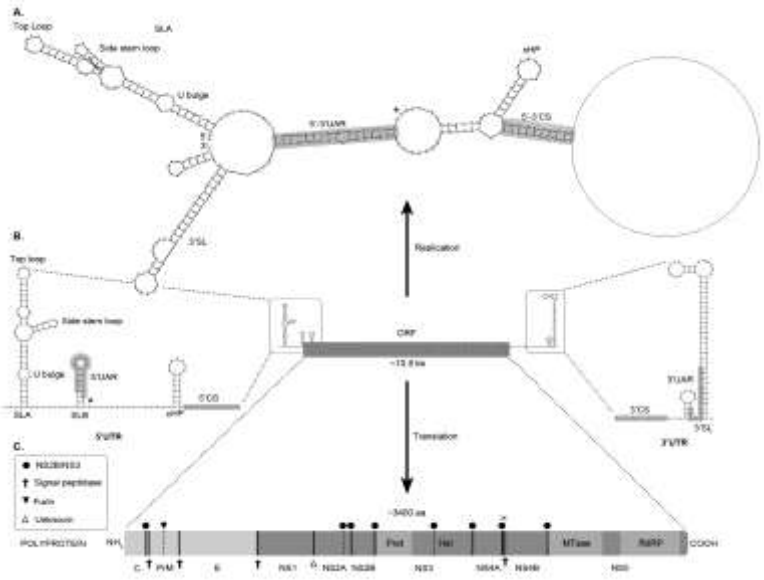

Figure 1. The DENV genome. A: schematic diagram of DENV genome circularization; B: organization of the 5' and 3'UTRs; C: polyprotein. A and B. The viral ssRNA(+) genome is ~11 kb long. The 5'UTR contains the large stem-loop A (SLA) the promoter for NS5 the RNA-dependent RNA polymerase (RdRP), followed by stem-loop B (SLB) that contains the 5' upstream AUG region (5'UAR) and the translation initiation codon (*). The 3'UAR is complementary to the 5'UAR. Another stem-loop hairpin structure (cHP) located within the C protein-coding region enhances selection of the 5' initiation codon. The 3'UTR contains conserved sequences such as the 3' stem-loop (3'SL) that includes the 3'UAR. The 3' cyclization sequence (3'CS) lies upstream of the 3'SL; it is complementary to the 5'CS present in the gene encoding the C protein. The predicted 5'-3'UAR and 5'-3'CS sequences are in grey, and hybridization between these regions is necessary for genome cyclization and RNA synthesis. C. The viral genome possesses one ORF coding for a polyprotein. The genes for the structural proteins are the capsid (C), the precursor of membrane protein (PrM) and E, followed by the genes for the NS proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). The polyprotein is processed in the cytoplasm by NS2B-NS3 (closed circles), by a signal peptidase (small arrow), or by an as yet unknown proteinase (open triangle) in the lumen of the ER, and prM is processed by furin (closed triangle) in the cellular secretory pathway.

In the region of the ORF encoding the capsid (C) protein, a stem-loop hairpin structure designated capsid hairpin (cHP) involved in codon start selection and viral RNA replication has been identified (Hahn *et al.*, 1987; Clyde and Harris, 2006), followed by a 5'cyclization sequence (CS) complementary to a 3'CS in the 3'UTR (Figure 1A and B) (Hahn *et al.*, 1987).

The ORF of over 10 kb comprises from its 5' to 3' end, information for the synthesis of three structural proteins C, prM and E, and the seven NS proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5) (Figure 1C), a feature common to all members of the genus *Flavivirus*. Yet size heterogeneity exists between the non-coding regions, particularly in the 3´UTR (Wallner *et al.*, 1995; Bryant *et al.*, 2005; Mendeiros *et al.*, 2007). The 5´UTR (Figure 1A, B) bears the stem-loop (SL) A, and the SLB that ends with the initiator AUG codon (Brinton and Dispoto, 1988). The SLA acts as promoter element recognized by NS5, which contains an RNA-dependent RNA polymerase (RdRp) domain for RNA synthesis (Filomatori *et al.*, 2006). It harbors a side stem-loop and a top loop, in addition to a U bulge (Lodeiro *et al.*, 2009) that are critical for DENV RNA replication. SLB contains upstream of the initiator AUG codon, a sequence known as 5′UAR (upstream AUG region), that is complementary to a sequence located at the 3′ end of the viral genome (Brinton and Dispoto, 1988; Alvares *et al.*, 2005a).

The 3´UTR (Figure 1A, B) includes three regions, a variable region immediately after the termination codon of the ORF followed by a core region, and finally a 3´-terminal region. The latter includes a conserved 3´SL of ~96 nt (Grange *et al.,* 1985; Brinton *et al.,* 1986; Mohan and Padmanabhan, 1991). The 3'SL contains a conserved 3'UAR sequence that is complementary to the 5'UAR located in the 5'UTR (Figure 1A) (Alvarez *et al.,* 2005a); distal flavivirus-conserved CACAG Box has also been described (Chen *et al.,* 1997; Filomatori *et al.,* 2006; reviewed in Markoff, 2003). The genome contains upstream of the 3'UAR, a conserved 3'CS (Hahn *et al.,* 1987) complementary to the conserved 5'CS and which together with the 5'UAR and 3'UAR is involved in RNA cyclization (Hahn *et al.,* 1987; Men *et al.,* 1996; Clyde and Harris, 2006). Moreover, a sequence located downstream of the initiation codon, designated 5' downstream AUG region (DAR) is involved in DENV replication (Friebe and Harris, 2010). Co- and post-translational cleavage of the DENV polyprotein is achieved by the viral-coded serine proteinase NS2B-NS3, by the cell convertase furin a proteinase of the trans-Golgi network (TGN), and by a cell signal peptidase (Falgout *et al.,* 1991; Cahour *et al.,* 1992; reviewed in Lindenbach and Rice, 2003). Polyprotein cleavage sites, conserved and unique motifs, important cysteine residues and potential glycosylation sites have been characterized (reviewed in Gubler *et al.,* 2007; Mukhopadway *et al.,* 2005). Table 1 provides a summary of the major activities so far identified for the viral proteins.

IV. CELL CYCLE OF DENV

A. Entry and fusion complex formation: E protein and cell receptors

Monocytes, macrophages, B and T lymphocytes, hepatocytes, endothelial cells, epithelial cells, DC and fibroblasts are all potential targets for DENV infection and replication, and viral antigens have been detected in liver, spleen, lymph node, thymus, kidney, lung, and skin cells (Upanan *et al.,* 2008). Hence the virus can replicate in a wide spectrum of cells, which might explain its ability to enter via various receptors, as discussed below. Receptor binding and membrane fusion characteristics of DENV entry have been studied in living cells by real-time fluorescence microscopy, and by direct biochemical or genetic analyses. Yet, the reports published on virus entry and on the molecules involved in this process, are controversial and may vary with the cell model used. Considerable evidence suggests that the first step in a primary infection is attachment of the viral glycoprotein E which is present on the surface of mature virions as a homodimer, to cell receptors on the surface of permissive cells (Fig. 2, step 1). The E protein consists of three domains. Domain I is in the N-terminal region and provides an organizational structure. Domain II or central domain is involved in dimerization and that contains a hydrophobic fusion loop essential for fusion of E to the cell membrane. Domain III in the C-terminal region is believed to be the receptor recognition and binding domain and has an immunoglobulin-like fold (reviewed in Chin *et al.,* 2007; Huerta *et al.,* 2008). The interaction of the E protein with the receptor(s) leads to a series of events on the viral particle and in the cell membrane, and also in the cytoskeleton allowing entry of the virus. Two types of cell receptors appear to be involved in facilitating entry of DENV into the human target cell, depending on the cell (reviewed in Huerta *et al.,* 2008). The first type corresponds to receptors of low affinity and specificity, including aminoglycan-type adhesion molecules such as heparan sulfate that are expressed in many cell types (Germi *et al.,* 2002; Lin *et al.,* 2002). The second type corresponds to lectin-type receptors such as DC-SIGN (dendritic cell-specific intercellular adhesion molecule 3-grabbing Table 1. Characteristics and functions of the DENV proteins

TABLE 1

Viral protein	Size (kDa)	Functions	References
M		-N- and C-termini have charged residues -Central hydrophobic region that associates with -Membrane -C-terminal hydrophobic sequence that acts as a signal -peptide for translocation to ER – Has nuclear localization signal and is found in the cytoplasm and nucleus; its role in the nucleus is unknown – Forms NCs with viral RNA; its dimerization triggered by viral RNA is required for virus assembly – Interacts with the apoptotic protein DAXX inducing apoptosis – Interacts with human Sec3, a member of the exocyst complex, a transcription and translation repressor of flavivirus that can retard infection	Wang et al. (2002), Ma et al. (2004) Ma et al. (2004) Markoff et al. (1997) Tadano et al. (1989), Wang et al. (2002), Bulich and Aaskov (1992), Tsuda et al. (2006), Sangiambut et al. (2008) Ma et al. (2004), Ku¨mmerer and Rice (2002), Kiermayr et al. (2004), Lopez et al. (2009), reviewed in Bartenschlager and Miller (2008) Netsawang et al. (2010) Bhuvanakantham et al. (2010)
pr		Precursor of membrane protein M, cleaved into pr þ M (26 þ 8 kDa) by furin located in TGN; required for infectivity – Liberated from polyprotein by host signalase located in the ER – Importance of His39 in the morphology of virus, and secretion and entry of virus into cell Interaction with E forms spikes on the virus surface, and is required for proper folding and secretion of E – Interaction with V-ATPase required for proper egress of virus – pr leaves virions after secretion of particles and exposure to neutral pH – By interacting with E, the pr peptide prevents fusion of E to cell membranes – pr blocks membrane fusion by binding to virus at acidic pH – Binds to the claudin-1 tight junction membrane protein that protects cells from the environment; interaction is required for virus entry	Elshuber et al. (2003) reviewed in Lindenbach and Rice (2003) Pryor et al. (2004) Duan et al. (2008), Li et al. (2008), reviewed in Lindenbach and Rice (2003) Duan et al. (2008) Li et al. (2008), Yu et al. (2008a) Zhang et al. (2003), Li et al. (2008), Yu et al. (2008a, 2009) Yu et al. (2009) Gao et al. (2010)
E	50	Intracellularly linked to prM forming heterodimers that protect E from premature acidification during transit through Golgi – Forms outer glycoprotein shell of virus – Major target for neutralizing antibodies – Cleavage of prM produces reversible conformational change in E – Protected by pr retained on particle from premature fusion to cell – Interaction with chaperones (Bip, calnexin, calreticulin) required for virus production	Guirakhoo et al. (1993), Konishi and Mason (1993), reviewed in Perara et al. (2008) reviewed in Lindenbach and Rice (1997, 2003) reviewed in Halstead (1988), Green and Rothman (2006) Li et al. (2008), Yu et al. (2008a) Li et al. (2008), Yu et al. (2008a) Limjindaporn et al. (2009)
		Specific amino acids determine hemorrhagic disease or encephalitis – Low pH in Golgi induces change in E structure important for formation of fusion complexes – Interacts with vacuolar-ATPase important for entry and egress of virus at low pH – E glycosylation decreases infectivity and increases virus release – Natural killer (NK) cell-activating receptor NKp44 interacts with E, leading to activation of NK cells and destruction of virally infected cells	Barker et al. (2009) reviewed in Heinz and Allison (2003) Duan et al. (2008) Lee et al. (2010), Duan et al. (2008) Hershkovitz et al. (2009) Limjindaporn et al. (2009) Hsieh et al. (2010)

		– Interaction with chaperones in ER facilitates folding and assembly of DENV proteins – Its transmembrane domain is responsible for retention and assembly of E on ER	
NS1	46	In flaviviruses, proposed to act early in replication cycle – May define pathogenesis – Possibly interacts with NS4A, thereby participates in viral RNA replication	Lindenbach and Rice (1997, 1999), Muylaert et al. (1997) Falconar (1997) Lindenbach and Rice (1999)
NS2	22	With NS4A and NS4B, is involved in IFN resistance and blocks IFN-b signal	
NS2B	14	Cofactor of NS3, forming serine proteinase activity in trans; acts in cis to cleave NS2B–NS3 – Required for proteolytic processing of DENV nonstructural proteins	Westaway et al. (2010) Falgout et al. (1991)
NS3b	70	Has N-terminal region with serine-proteinase acivity and C-terminal region with NTPase, RNA helicase, and 5' RNA triphosphatase activities – Cleavage activity retained in NS2B–NS3 heterodimer – NS5 stimulates NS3 activities – Required for proteolytic processing of DENV nonstructural proteins – Binds to La protein	Wengler and Wengler (1993), Li et al. (1999), Sampath et al. (2006), Luo et al. (2008a,b) Leung et al. (2001), Bera et al. (2007) Yon et al. (2005) Falgout et al. (1991) Garcia-Montalvo et al. (2004)
NS4	16	C-terminal region known as 2K fragment; involved in regulating membrane rearrangements – Associates with membranes through its hydrophobic regions – Probably involved in RNA replication – Induces membrane rearrangements resembling virus-induced structures	Zou et al. (2009), Miller et al. (2007) reviewed in Lindenbach and Rice (2003) Miller et al. (2007)
NS4B	27	Blocks IFN-induced signal-transduction cascade – Interacts with NS3 and dissociates it from ssRNA – Downregulates expression of STAT2 – Participates in formation of RCs	Munoz-Jordan et al. (2003) Umareddy et al. (2006) Jones et al. (2005) Miller et al. (2006)
NS5	103	Has MTase (N-terminus) and RdRp (C-terminus) motifs – Possesses two nuclear localization signals for transport to nucleus – Is a nuclear phosphoprotein; only hypophosporylated NS5 is located in cytoplasm where it interacts with NS3 – Modulates enzymatic activities of NS3 – Possesses a nuclear export signal and interacts with CRM1 for export to cytoplasm – Participates in methylation of viral RNA cap structure – Binds to La protein – Its interaction with STAT2 required for IFN signaling, reduces level of expression of STAT2. Inhibits IFN-a signaling by binding to the STAT2 and inhibits its phosphorylation	Ackermann and Padmanabhan (2001), Egloff et al. (2002), Yap et al. (2007); Selisko et al. (2010) Brooks et al. (2002) Kapoor et al. (1995) Rawlinson et al. (2009) Yon et al. (2005) Rawlinson et al. (2009) Egloff et al. (2002), Geiss et al. (2009) Garcia-Montalvo et al. (2004) Ashour et al. (2009), Mazzon et al. (2009)

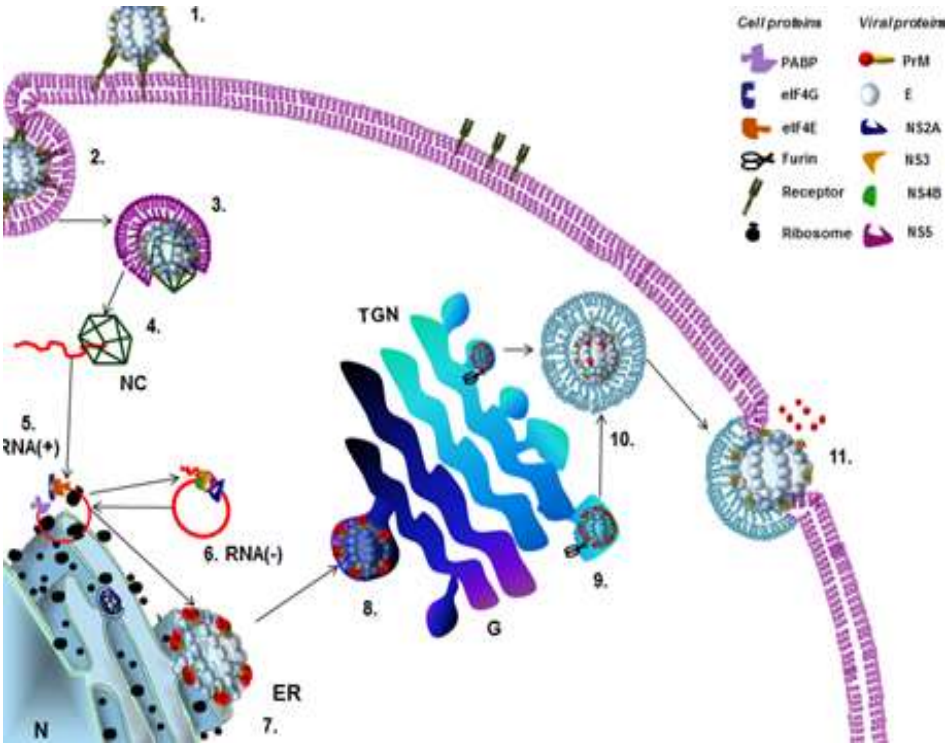

Figure 2. Schematic representation of the DENV life cycle. The DENV E protein is the major component of the virion surface. 1. Attachment: The initial step in the viral life cycle is attachment of the E protein to a cellular receptor (forming a fusion complex) such as heparan sulphate, a mannose-specific C-type lectin, but a specific receptor for internalization of the virus into host cells has not been identified. 2. Endocytosis: Following receptor binding, the virus is internalized into the cells through clathrin-mediated endocytosis transporting the virus particles to endosomes 3. Fusion of membranes: In the cytoplasm, acidification of the endosome lumen induces structural changes in E and promotes fusion between the virus particle and the endosomal membrane. 4. Uncoating: A fusion pore is formed, the nucleocapsid (NC) is delivered into the cytoplasm, and after uncoating the viral RNA (red) is released from the NC into the cytoplasm. 5. Translation: The RNA(+) is directly translated as a single endoplasmic reticulum (ER)-bound polyprotein. The 5' cap structure of the viral mRNA promotes assembly of eukaryotic initiation factors (eIFs) such as eIF4E and eIF4G and recruits ribosomes on the mRNA. In addition, in spite of the fact that the mRNA lacks a poly(A), the poly(A)-binding protein (PABP) interacts with the 3'UTR, suggesting that the viral genome is circularized though PABP-eIF4G interaction. The polyprotein is processed by viral and cellular proteases into three (then four) structural proteins and seven NS proteins (some of which are highlighted). 6. Replication: The NS proteins (highlighted) actively replicate the viral RNA(+) in replication complexes (RCs) associated with cellular membranes, producing complementary RNA(-), which in turn is used as template to produce RNA(+), that functions as genomic RNA. 7. Assembly: Following RNA replication and translation, virus assembly is achieved when one copy of RNA interacts with several copies of C protein forming NCs that are enveloped by the heterodimer prM-E, to assemble into immature virus particles that bud into the lumen of the rough ER. 8 and 9. Maturation: Virus particles transit through the Golgi (G) and the trans-Golgi network (TGN) where prM is cleaved by cellular furin resulting in the formation of particles containing the pr, M and E proteins. 10 and 11. Exocytosis and Release: The mature virus particle migrates to the cell membrane and is released from the cell as is also the pr protein. N: nucleus.

non-integrin) expressed in some antigen-presenting cells such as immature DCs (Tassaneetrithep *et al.*, 2003; Lozach *et al.*, 2005). These molecules presumably concentrate viral particles on the cell surface

(Tassaneetrithep *et al.*, 2003; Lozach *et al.*, 2005; Reyes del Valle *et al.*, 2005; reviewed in Huerta *et al.*, 2008), and facilitate recognition of the second type of receptors, such as the heat shock proteins (HSP) 70 and 90 (HSP70 and HSP90). This interaction induces the formation of endosomes; this phenomenon designated "receptor-mediated endocytosis" (Fig. 2, step 2) allows virus entry into the cell (Reyes del Valle *et al.*, 2005). However, the implication of HSP70/90 as receptors is still a matter of debate and could depend on the cell types examined (Reyes-del Valle *et al.*, 2005; Cabrera-Hernandez *et al.*, 2007; Chavez-Salinas *et al.*, 2008). Both these proteins are associated with microdomains, whose interaction is important for DENV entry. Interestingly, HSP70/90 have been postulated to be implicated in the entry of other viruses such as *Hepatitis E virus* (Zheng *et al.*, 2010) and flaviviruses (Lin *et al.*, 2007; Ren *et al.*, 2007). Certain proteins known as adaptor proteins such as GRP78 are believed to also be part of the DENV entry complex but their functions remain undefined (Cabrera-Hernandez *et al.*, 2007). Associations between E and adaptor proteins are designated here "fusion complexes". The presence of lipid rafts or coated pits in virus fusion complexes indicates that vesicle formation depends on cholesterol (in human cells) or clathrin (in mosquito cells) (Lee *et al.*, 2008a; Acosta *et al.*, 2008; van der Schaar *et al.*, 2008). Together these results show that regardless of the receptor used, at least two mechanisms of flavivirus entry into insect and mammalian cells exist: virus entry can occur by direct fusion of the virus to the cell membrane or by clathrin-dependent endocytosis (Hase *et al.*, 1989a; Hase *et al.*, 1989b; Lim and Ng, 1999; Se-Thoe *et al.*, 2000; Chu and Ng, 2004; Chu *et al.*, 2006; Krishnan *et al.*, 2007; Suksanpaisan *et al.*, 2009). Based on experiments in which it was shown that ablation of clathrin-mediated endocytosis only reduces virus entry (Chu and Ng, 2004; Chu *et al.*, 2006; Krishnan *et al.*, 2007), another independent pathway of entry was proposed (Suksanpaisan *et al.*, 2009). However, it should be stressed that different cell types were used in the various experiments referred to above.

Another mode of entry for DENV is by ADE (see Section II. The dengue problem. D. Pathogenesis) (Kou *et al.*, 2008). It has been demonstrated that infection of U937 monocytes is induced by ADE-mediated FcγRI, as is also infection of K562 leukaemic cells by FcγRII (Rodrigo *et al.*, 2006). Although the role of FcγRIII in ADE-DENV infection remains unclear, it was recently reported that the immune tyrosine activation motif of FcγRIII RIIIA is essential to mediate ADE (Moi *et al.*, 2010). Interestingly, in mature DCs but not in immature DCs expressing high levels of DC-SIGN, ADE requires the Fcγ receptor IIa (Boonnak *et al.*, 2008). Finally, lipid rafts are necessary for AD-mediated infection of U937 cells by DENV (Puerta-Guardo *et al.*, 2010).

B. Virus adsorption and fusion to the endosome membrane

Evidence obtained to date suggests that the pathway of DENV entry after attachment to the host cell is primarily by clathrin-dependent endocytosis (Fig. 2, step 2). At pH 6.2-6.4, homodimer dissociation is facilitated and the resulting E monomers present a fusion loop that anchors to the endosome membrane, catalyzing the formation of E protein homotrimers (Heinz *et al.*, 2004). This association induces a bend in the endosomal membrane promoting formation of a bridge (hemifusion stalk) between the virus and the endosomal membrane, leading to fusion. Thus, the nucleocapsid (NC) is released into the cytoplasm (Fig. 2, steps 3 and 4); how this occurs is largely unclear. After uncoating, the DENV genome is poised for translation by the host-cell machinery.

Although the processes leading to fusion complex formation and to the E conformational changes involved in membrane fusion are established, no satisfactory agreement has been reached as to whether early and/or late endosomes are the site where viral fusion complexes form (Krishnan *et al.*, 2007). Using HeLa cells, chemical inhibitors and small interfering RNAs (siRNAs), it was demonstrated that Rab 5 (Rabaptin-5), a small GTPase protein that is a marker for early endosomes, showed that early endosome components are indispensable for virus amplification (Krishnan *et al.*, 2007).

C. Intracellular transport of the viral genome

Little is known about the mechanisms involved in the disassembly of the NCs, the transport of incoming genomes to the rough endoplamic reticulum (RER), or the recruitment of ribosomes by genome RNA. Intracellular transport of NCs/genomes is mediated by direct interaction with the cytoskeletal transport machinery (reviewed in Döhner and Sodeik, 2005; Kanlaya *et al.*, 2009). The cytoskeleton is composed of actin and vimentin filaments, microtubules (MTs) and intermediate filaments (IFs) that are constantly modified during the cell cycle. These modifications include morphological changes and activation, *i.e.* maturation and rearrangements that can be enhanced by the viral pathogen (Chen *et al.*, 2008).
DENV can alter the components of the cytoskeleton, but how this is achieved is poorly understood; it requires actin-rich regions endowed with a dynamic reorganization mechanism (Chen *et al.*, 2008; Talavera *et al.*, 2004) and also affects vimentin filaments, MTs and IFs. In the presence of DENV the arrangement is modified and the vimentin filaments retract from the cell periphery and from around the nucleus (Chen *et al.*, 2008). Other studies showed that infection of human microvascular endothelial cells-1 (HMEC-1) with DENV-2 promotes actin reorganization, regulated by the GTPase proteins, ras-related c3 botulinum toxin substrate 1 (Rac1) and cell division cycle 42 (Cdc42); this is critical for the formation and function of filopodia, necessary for viral entry (Zamudio-Meza *et al.*, 2009).
Thus the cytoskeletal transport machinery may facilitate the transfer of the viral genome to the ribosomes. Yet the mechanism involved and the time necessary for capsid disassembly and RNA release are unknown.

D. Genome expression

Liberated from its structural proteins, the DENV genome (Figure 1) becomes accessible to perform its three major functions: 1) as mRNA for the synthesis of the viral polyprotein (translation), 2) as template for the synthesis of further RNA strands (replication), and 3) as genome incorporated into new viral particles (encapsidation). It is important to bear in mind that DENV genome translation is coupled to replication since the viral genome must be translated to synthesize the NS proteins required for RNA replication (reviewed in Harris *et al.*, 2006). Translation of the DENV RNA(+) produces a single endoplasmic reticulum (ER)-bound polyprotein (Fig. 2, step 5). Synthesis of the viral polyprotein is initiated at the 5' proximal AUG codon in the RNA. The cap structure of the viral mRNA facilitates assembly of eukaryotic initiation factors (eIFs) such as eIF4E and eIF4G and recruits ribosomes on the mRNA. Interestingly, the poly(A)-binding protein (PABP) can bind to the 3'UTR (Polacek *et al.*, 2009b) even though the genome lacks a poly(A) tail, suggesting that circularization of the genome via interaction with the PABP-eIF4G complex could be required for efficient translation (Figure 2 step 5).
Interestingly, initiation of DENV mRNA translation can also occur efficiently when cap-dependent translation is inhibited, such as when the cap-binding protein eIF4E is limiting (Edgil *et al.*, 2006). Experiments performed in the presence of inhibitors of cellular capped mRNA translation, showed that DENV RNA or a reporter mRNA flanked by the DENV 5'UTR and 3'UTR was efficiently translated. Translation by a cap-independent initiation mechanism may facilitate amplification of viruses that multiply in divergent hosts (*e. g.* mosquitoes and humans), and may also help the virus accommodate to various intracelular environments. The highly conserved 3'SL structure present in the 3'UTR (Figure 1B) facilitates mRNA binding to polysomes and is a major player in promoting efficient DENV-2 mRNA translation (Holden and Harris, 2004); during the first round of translation it stimulates translation in the absence of viral proteins. Natural variants of DENV-2 that differ in their ability to multiply in primary human cells appear to vary in their ability to synthesize viral proteins *in vivo* and *in vitro* (Edgil *et al.*, 2003). These differences are linked to mutations in the 3'UTR and sugget that other regions than the coding sequence are important for efficient translation and could be activated by modifying either RNA-RNA or RNA-protein interactions. Certain cell proteins bind to the 3'UTR. Indeed, in addition to certain translation initiation factors important for cap-dependent DENV RNA translation, the 3'SL binds several cell proteins such as the eukaryotic elongation factor (eEF) 1A enhanced viral RNA transcription (Davis et al., 2007). Other reports have shown that the La autoantigen, the polypyrimidine-tract-binding protein

and the Y-box-binding protein-1 bound to the 3'UTR (De Nova-Ocampo *et al.*, 2002; Garcia-Montalvo *et al.*, 2004; Davis *et al.*, 2007; Paranjape and Harris, 2007), but the functional roles of these interactions in replication and/or translation have not been clearly established. Some results have proposed that translation and subsequent replication occur within intracellular membranes structures, whose origins are not well defined (reviewed in Clyde *et al.*, 2006; Miller and Krijnse-Locker, 2008).

E. Genome replication

The association of the machinery for DENV negative-sense RNA [RNA(-)] synthesis with host intracellular membranes is poorly understood, but the membranes probably play a structural and organizational role in forming the replication complex (RC). However, it is known that following translation, viral NS proteins together with host factors form the RC responsible for the synthesis of RNA(-) complementary to the RNA(+), resulting in an intermediate double-stranded (ds) RNA. The complete DENV RNA replication cycle includes the synthesis of RNA(-), and of new RNA(+) that functions as mRNA (Fig. 2 step 6), and as genomic RNA to form new virus progeny. RNA synthesis is recgulated by sequences located in the 5´and 3´UTRs: the 5'-3'CS and 5'-3'UAR sequences (Fig. 1A, B) mediate Mg^{2+}-dependent and cell protein-dependent circularization resulting in long-range RNA-RNA interactions important for DENV RNA replication (Khormykh *et al.*, 2001; Lo *et al.*, 2003; Alvarez *et al.*, 2005;. Circularization is further facilitated by the presence of a 6 nt-long 5'DAR sequence (Friebe and Harris, 2010). Although the specific role of NS3 in replication remains unclear, its helicase domain could be involved in unwinding RNA secondary structures present in the 3'UTR and assisting the first steps of replication. NS3 is a multifunctional protein with RNA-stimulated nucleoside triphosphatase (NTPase), ATPase/helicase, and RTPase (Yon *et al.*, 2005; Sampath *et al.*, 2006; Wang, *et al.*, 2009a) activities that are essential for viral RNA replication and capping. SLA at the 5' end of the 5'UTR is the promoter for the RdRp activity of NS5 and RNA synthesis (Filomatori *et al.*, 2006). In transfected cells, NS5 interacts with the 5' SLA, but surprisingly not with the 3'UTR (Filomatori *et al.*, 2006). Various structures in the 5'SLA (UAR, cHP and CS) are involved in circularization of the DENV genome and in promoting RNA synthesis (Friebe and Harris, 2010; Lodeiro *et al.*, 2009). Despite sequence and structural differences between the 5'SLAs of DENV-1 and DENV-2, the RdRp of DENV-2 efficiently uses the 5'SLA of DENV-1 for RNA replication (Hahn *et al.*, 1987; Filomatori *et al.*, 2006; Yu *et al.*, 2008b). Thus the requirement for the homologous 3´SL for viral replication is related to specificities of other viral NS proteins in addition to NS5 which not only functions as the RdRp, but also acts as the MTase implicated in capping of the viral RNA. Alternatively, the specificity of NS5 could be altered by its interaction with other NS proteins or with cell proteins. A single mutation within the 5'UAR can decrease RNA synthesis up to 3000 fold during DENV genome replication (Alvarez *et al.*, 2008). In addition, genomes with disruption of the stem of SLB by mutations that still maintain complementarity with the 3'UAR, can be translated and replicated. Therefore, the 5'UAR sequence acts as a *cis*-element regulating DENV RNA replication. In the DENV genome, interaction between the 3' and 5' CSs also facilitates hybridization of the 5'-3'UARs, that form a 15 nt-long base-paired structure interrupted by a C bulge and a G-G mismatch (Fig. 1B) (Alvarez *et al.*, 2008). Although hybridization between the 5´ and 3´UARs requires interaction between the 5' and 3'CSs, interaction between the 5' and 3'CSs does not depend on interaction between the 5' and 3'UARs (Polacek *et al.*, 2009a). Studies performed *in vitro* using viral subgenomic (sg) RNA templates containing the 5´ and 3´CSs showed that interaction between the two terminal regions is necessary for the synthesis of RNA(-) from RNA(+) (Ackermann and Padmanabhan, 2001; You *et al.*, 2001; Nomaguchi *et al.*, 2004). Recently, the presence of a small sgRNA derived from the 3'UTR by cell nuclease digestion and essential for virus-induced pathogenicity, was identified in DENV-infected cell cultures and in animal tissues (Pijlman *et al.*, 2008; Liu *et al.*, 2010). Both the sgRNA and genomic RNA accumulated together during viral RNA replication. It will be interesting to define the function of this non-coding sgRNA in the virus life-cycle. The 3´UTR(-) could act as a promoter element for the synthesis of new RNA(+) as does the 3'UTR(+) for the synthesis of RNA(-). In addition, the 3'UTR(-) of DENV

interacts with La, a nuclear protein that participates in cell mRNA transcription initiation, forming a ribonucleoprotein complex in association with other cell proteins such as calreticulin and the protein disulfide isomerase (Yocupìcio-Monroy *et al.*, 2003).
Circularization of the viral RNA favors coupling of translation and replication. This occurs in the cytoplasm on induced cell membranous structures probably derived from the ER, and in vacuoles induced by the virus and known as viral RCs. NS2A in addition to playing a direct role in viral RNA replication, could also be the viral protein that targets the viral RCs to membrane organelles (Mackenzie *et al.*, 1998). NS4B which presents at its C-terminus a highly hydrophobic fragment (designated 2K) that function as a signal sequence, serves for the translocation of NS4B into the ER lumen, where it is a component of RCs (Miller *et al.*, 2006). In the membrane-bound viral RCs, NS4B is associated with NS3 and dsRNA, a marker for replicating viral RNA. During this process, proteolytic removal of a 2 kDa peptide from NS4A is important for the induction of membrane alterations that may harbour the viral RCs (Miller *et al.*, 2007). Dissociation of NS3 from ssRNA by NS4B *in vitro* enhances the NS3 helicase activity, suggesting that NS4B can modulate DENV replication (Umareddy *et al.*, 2006). Viral genome replication takes place in the RCs (reviewed in Bartenschlager and Miller 2008), although some replication activity has been detected in the nucleus (Uchil *et al.*, 2006). The question however remains of how the RCs are formed. A role of authophagy in virus replication has recently become a new emerging field of investigation. Autophagy develops in an area of the cytoplasm where a membrane pre-exists, and expands the membrane to form double-membrane vesicles; it is part of a lysosomal degradation pathway that is important for cell remodeling and development, and that is also involved in various disease processes. DENV appears to also induce authophagy as a means of enhancing replication in the cell (Colombo, 2005; Lee *et al.*, 2008b), and viral RNA replication has been associated with double-membrane structures (Uchil *et al.*, 2003; reviewed in Miller and Krijnse-Locker, 2008) that are hallmarks of autophagosomes (Dunn, 1990). Other studies showed that the induction of autophagy by DENV-2 and DENV-3 resulted in an increase in the titers of extracellular and intracellular virus (Khakpoor *et al.*, 2009). Moreover, DENV-2 triggers autophagy by increasing the expression of LC3-II (light chain 3 form II of the microtubule-associated protein 1) and green fluorescent protein (GFP)-LC3 dot formation. In addition, LC3-II is associated with autophagosome membranes, and co-localizes with viral dsRNA, NS1, and the ribosomal protein L28 (Kuma *et al.*, 2004; Panyasrivanit *et al.*, 2009b). Inhibition of fusion of autophagosomes and amphisomes with lysosomes decreases DENV-3 production, suggesting a role for authophagosomes in the DENV life cycle (Panyasrivanit *et al.*, 2009a). Based on results showing that DENV components colocalize with markers of autophagic and endosomal vesicles, it was proposed that amphisomes constitute a site of DENV genome replication and translation. The activation of the cell autophagy machinery to promote DENV RNA replication depends on the ATG5 protein, an initiator of autophagosome formation during autophagy progression (Kuma *et al.*, 2004; Lee *et al.*, 2008b). Inhibition of autophagy by ATG5 knockout or treatment of cells with lysosomes that inhibit fusion of autophagosomes and amphisomes, is associated with a decrease in DENV replication and reduced progeny virus particle formation (Limjindaporn *et al.*, 2009; Khakpoor *et al.*, 2009). Interestingly, there appears to be a link between DENV entry and replication/translation in terms of a continued association of the virus life cycle with membranes of an endosomal-autophagosomal lineage (Panyasrivanit *et al.*, 2009a). Thus, DENV replication/translation is coordinated by *cis*-acting genomic sequences, viral proteins and cell factors. Future studies should clarify how autophagic membranes and how DENV coordinates the authophagic processes.

F. Maturation and release of DENV from the host cell

After polyprotein synthesis and genome replication have occurred, the next step leading to viral particle formation is encapsidation. Although the first indications of interaction between the viral RNA and C were demonstrated over a decade ago (Khromykh *et al.*, 1999), the precise mechanisms involved in RNA-C complex (or NC) formation are unclear. Recently, Samsa *et al.* (2009) reported that mature C protein is

associated with lipid droplets (LDs), defined as ER-derived organelles located in the cytoplasm and that contain various proteins surrounded by a core of neutral lipids and a monolayer of phospholipids; this association is triggered by hydrophobic residues present in the center of C (Ma *et al.,* 2004). Infection of cells by DENV stimulates the formation of LDs, and decreasing the number of LDs reduces virus replication. Strong evidence suggests that viral RNA-C protein assembly occurs in membrane structures derived from the ER (Fig. 2, step 7), forming NCs. In addition to its RNA-binding motifs, the N-terminal region (aa 21-100) of the C protein contains four hydrophobic α helices essential for association with the ER (Ma *et al.,* 2004). Thus, the partially assembled virus NCs, bud from the lumen of the ER (Fig. 2, step 7), and become enveloped by a lipid membrane that contains in addition to NCs, the two structural proteins, prM and E, in the form of prM-E heterodimers (Mukhopadhyay *et al.,* 2005, Wang *et al.,* 2009b) that are incorporated into immature virions by interaction with the NC; this step constitutes the assembly of immature viral particle. Yet NCs are not prerequisites for the formation and secretion of viral particles since sub-viral particles composed only of prM and E are also secreted from DENV-infected cells (Mukhopadhyay *et al.,* 2005). The E protein undergoes reversible pH-dependent conformational changes during egress of virions through the secretory pathway. In the immature state the prM-E heterodimers are grouped as trimers, whereas in the mature particles there are E homodimers, and E forms homotrimers when the virus fuses with the host cell endosomal membrane (Bressanelli *et al.,* 2004). During this transport process the virus particles pass through the trans-Golgi Network (TGN) where the prM protein is cleaved by the protease furin but the pr segment continues to be associated with the virion until the virion is released from the cell. The retention of the pr segment prevents premature binding of the E protein to the exosomal membrane during transit of the virus in the acidic enviroment of the TGN. These mature viral particles are released from the TGN into the cytoplasm and are transported to the outer cell membrane by exocytosis (Fig. 2, steps 10) (Yu *et al.,* 2008a; Yu *et al.,* 2009; reviewed in Perera and Kuhn, 2008). Finally the cleaved pr and virions are released into the extracellular medium upon particle secretion (Figure 2 step 11) (Yu *et al.,* 2009). Virus maturation in the lumen of the TGN produces mature virions composed of one copy of RNA, 90 homodimers of E and 180 copies of prM. The transition from immature (spiky) to mature (smooth) particles due to the cleavage of prM occurs during transit of the particles through the Golgi into the TGN (Fig. 2, steps 8 and 9)(Wang *et al.,* 2009b). Cleavage of prM to M by furin is essential for DENV infectivity (Zybert et al., 2008). Indeed, treatment of immature viruses with exogenous furin restores viral infectivity. Analyses performed with other flaviviruses suggest that prM cleavage is required for full maturity and infectivity of the virus particles (Wengler and Wengler 1989). However, cleavage of DENV prM is inefficient, as various cell types infected with DENV-2 release large amounts of virions with unprocessed prM (Elshuber *et al.,* 2003). In addition to preventing fusion of E to the exosomal membrane (Yu *et al.,* 2008a), pr retention could also 1) constitute a mechanism favoring flavivirus trafficking and stability in the cell secretory pathway, 2) be required for interaction with the vacuolar-ATPases (V-ATPases) whose silencing leads to reduction in DENV replication, and 3) establish a suitable pH environment for efficient virus secretion (Duan *et al.,* 2008). As mentioned above, acidification of intracellular organelles such as components of the secretory pathway is crucial for infectivity (Yu *et al.,* 2008a; Yu *et al.,* 2009). Previous reports have shown that V-ATPases are multisubunit enzymes that acidify various organelles including lysosomes and components of the secretory pathway, facilitating protein processing and acid-dependent protein degradation during DENV infection (Duan *et al.,* 2008). Residues 76 to 80 of prM participate in the interaction with V-ATPases, and the V-ATPase-virus interaction is critical for efficient virus entry and egress. Thus, the viral particles assemble and bud from the lumen of the ER, and virus maturation occurs in the TGN; finally the mature virus particles are released by exocytosis. In addition, it was recently demonstrated that the human immunoglobulin heavy chain binding proteins, calnexin and calreticulin, all ER-resident chaperones, interact with the E protein (Limijindaporn *et al.,* 2009). Silencing of the three corresponding genes using siRNAs, affected the production of infectious DENV suggesting that these chaperones participate in the folding and assembly of the viral proteins. The E protein of DENV is glycosylated in the residues 67 and

153/154. Loss of either of the two E protein glycan enhanced infectivity of variants for mosquito cells and reduced virus releases in mammalian (Hanna *et al.,* 2005; Lee *et al.,* 2010).

V. siRNAs AND NEW STRATEGIES TO CONTROL DENV REPLICATION

Understanding the cellular cxomponents involved in DENV infection is imperative as it should allow better comprehension of dengue pathogenesis, and lead to the elaboration of gene-targeted inhibitor strategies. However, only a few reports of inhibitory factors of DENV infection have appeared. Lately, siRNAs have been used *in vivo* as a gene silencing approach to decrease DENV replication. dsRNA is cleaved by Dicer, an RNAse-III-type enzyme, into 21-25 nt-long siRNAs. siRNAs have been used as a knockdown mechanism to study cellular and viral genes involved in virus replication and constitute a new pathway for clinical treatment of various infectious diseases. Several reports have demonstrated the efficiency of the siRNA strategy to block DENV production (Ang *et al.,* 2010; Mukherjee and Hanley, 2010; Subramanya *et al.,* 2010). The nucleotide sequence of the 3'UTR which is common to the genome of all four DENV serotypes, was used to design siRNAs and these siRNAs silenced DENV RNA replication. Adeno-associated virus encoding siRNAs targeted to the 3'UTR of the DENV RNA also reduced DENV infection in Vero cells and in human DCs in a dose-dependen manner (Zhang *et al.,* 2004). Moreover, infection of human dermal microvascular endothelial line-1 cells with DENV-2 induces high expression of β3 integrin, and pre-incubation of the virus with soluble αβ integrin or silencing of the β3 integrin gene using siRNAs, drastically reduces virus entry (Zhang *et al.,* 2007). In Huh7 cells, the use of siRNAs targeting genes implicated in clathrin-mediated endocytosis inhibited DENV entry into target cells (Ang *et al.,* 2010). In HeLa cells, silencing of the gene encoding the V-ATPase, a proton pump that is key to establishing the low pH of endosomal compartments resulted in decreased DENV replication (Krishnan, *et al.,* 2007), demonstrating the requirement of V-ATPase in endosomal DENV entry. Moreover, knockdown of the Rab 5 GTPase gene by siRNAs revealed that DENV infection requires the expression of Rab 5, a key regulator of transport to early endosomes. Cholesterol is an important element in DENV infection; its depletion with methyl-ß-cyclodextrin or its chelation with filipin III, a member of the polyene family of antibiotics, diminishes entry of DENV or Japanese encephalitis virus (Lee *et al.,* 2008a). Furthermore, selective silencing of enzymes involved in cholesterol biosynthesis using siRNAs inhibits DENV replication in A549 cells (Rothwell *et al.,* 2009). Both studies suggest that cholesterol modulation affects DENV replication, although the mechanism(s) whereby this occurs is as yet unknown. DENV replication occurs in association with the ER, where the viral particles assemble; in a search for the role of host ER chaperones in these processes, it was demonstrated that siRNAs that separately silence three ER-resident chaperones, the immunoglobulin heavy chain-binding protein known as BiP, calnexin or calreticulin, significantly decreased the yield of infectious virus production (Limjindaporn *et al.,* 2009). Moreover, using image-based immunofluorescence assays, inhibitors of the c-Src protein kinase were shown to be potent inhibitors of DENV replication (Chu and Yang, 2007), preventing virus assembly within the RC. The data suggest that siRNAs could be used to hinder DENV infection and could serve as therapeutic strategy.

Interestingly, infection by DENV-2 of cultured *A. aegypti* cells, or DENV-2 administered orally to adult mosquitoes, led to the production of siRNAs specific for the virus, whereas impairing the siRNA machinery increased virus production in this vector (Sánchez-Vargas *et al.,* 2009). Infection of *Drosophila melanogaster* S2 cells with any of the four serotypes of DENV induced siRNA production (Mukherjee and Hanley, 2010). Knockdown of one gene implicated in the RNA interference (RNAi) machinery led to enhancement of viral replication. Consequently, infection of insect cells activates an antiviral response mediated by siRNAs.

VI. CONCLUSIONS

In recent years, new approaches such as transcriptomic/proteomic and microarray techniques to study integral aspects of virus life cycles have led to more profound knowledge concerning the relationship between virus and host. It was known that formation of infectious DENV requires the concerted action of viral structural proteins. More recently the importance for viral genome expression of viral NS proteins and cell proteins, and of regions of the viral genome that lie within the ORF are being recognized, as is also the coupling of viral RNA replication and virus assembly. These approaches have also demonstrated that NS proteins participate in the localization of active RCs and mediate formation of specialized assembly sites to regulate the release of RNA from the RCs and RNA packaging in the budding virus. Although replication occurs in the cytoplasm, NS5 has also been immunolocalized in the nucleus where its specific role in the virus life cycle appears to be to antagonize the antiviral response. However the functions of other DENV proteins that traffic between the nucleus and cytoplasm are unclear. Likewise, the function of the C protein in the nucleus is unknown. These observations nevertheless illustrate the essential roles of the viral proteins and their use as potential targets for genetic therapeutic strategies against DENV infection. The identification of host cell factors important for DENV infection remains an essential but largely unexplored area. Although the functions of the NS proteins in the production of infectious progeny are beginning to emerge, further studies should aim at elucidating their specific mechanisms of action so as to exploit this knowledge in the development of highly efficient antiviral drugs for dengue treatment.

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Chapter 2

Viral Recognition by the Innate Immune System:The Role of Pattern Recognition Receptors

Silvia Torres Pedraza, JuanGuillermo Betancur, and Silvio Urcuqui-Inchima

Pattern recognition receptors are the main sensors of the innate immune response. Their function is to recognize pathogen-associated molecular patterns, which are molecules essential for the survival of microbial pathogens, but are not produced by the host. The recognition of pathogen-associated molecular patterns by pattern recognition receptors leads to the expression of cytokines, chemokines, and co-stimulatory molecules that eliminate pathogens, such as viruses, for the activation of antigen presenting cells and for the activation of specific adaptive immunity. Among the most thoroughly studied pattern recognition receptors implicated in viral infections, there are the toll-like receptors (TLRs) and the RNA helicase-type retinoic acid- inducible gene-I receptors [or RIG-like receptors (RLRs)]. Moreover, other proteins such as PKR, 2'-5' OAS, and ADAR also act as effector proteins in antiviral responses. The identification and characterization of pattern recognition receptors have contributed to our knowledge of the role of innate immunity in viral infections and has led us to better understand host-pathogen interactions. The most recent findings concerning the role of TLRs and RLRs in viral infections, the molecular mechanisms of viral ligand recognition through pattern recognition receptors, and the activation of their signaling pathways are discussed in this review

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Innate immunity is the first protecting barrier of the host against foreign agents; its response and quality determine the development of adaptive immunity responses. The activation of the innate immune response involves a wide spectrum of cells and soluble factors that recognize and exert effector functions when challenged by pathogens that can enter the host. However, it was the description of pathogen recognition receptors (PRRs) and, particularly, of Toll-like receptors (TLRs) that led to understanding

Table 1	
Viral recognition by PRRs	
Receptor	**Ligand/Viruses**
Intracellular Recognition Receptor RIG-I	Paramyxovirus Rabies virus Dengue virus Japanese encephalitis virus Hepatitis C virus Influenza virus West Nile virus
MDA5	Encephalomyocarditis Virus Varicella-zoster Virus
Extracellular/endosomal sensors	
TLR2	Lipoteichoic acid Lipophosphoglycan (LPG) Glycophosphatidylinositol (GPI) Hemagglutinin protein of the Varicella-zoster Virus herpes Simplex Virus-1 Human Cytomegalovirus
TLR4	LPS Syncytial Respiratory Virus Envelope protein of the Mouse Mammary Tumor Virus
TLR3	Poly (I:C) murine cytomegalovirus vesicular stomatitis virus lymphocytic choriomeningitis virus reovirus West Nile virus
TLR7/8	R848 Imiquimod Loxorbine Human Immunodeficiency Virus Vesicular Stomatitis Virus Dengue Virus Influenza Virus
TLR9	Synthetic CpG-containing DNA Vesicular Stomatitis Virus Murine Cytomegalovirus Herpes Simplex Virus -1 and 2

the prominent role that innate immunity plays in the recognition of microbial pathogens and in the regulation of the immune system1.One of the most interesting aspects of innate immunity is the wide range of molecules recognized by PRRs (C. A. Janeway Jr & Medzhitov, 2002). These receptors recognize diverse structural components or ligands (Table 1)2, essential for the survival of microorganisms, known as pathogen-associated molecular patterns (PAMPs) (Palm & Medzhitov, 2009). Recognition of PAMPs by PRRs stimulates an intracellular signaling cascade that involves the activation of diverse transcriptional factors involved in regulating expression of cytokines. Such cytokines play important roles in host protection, in the activation and migration of antigen presenting cells, and in the induction of the adaptive immune response.

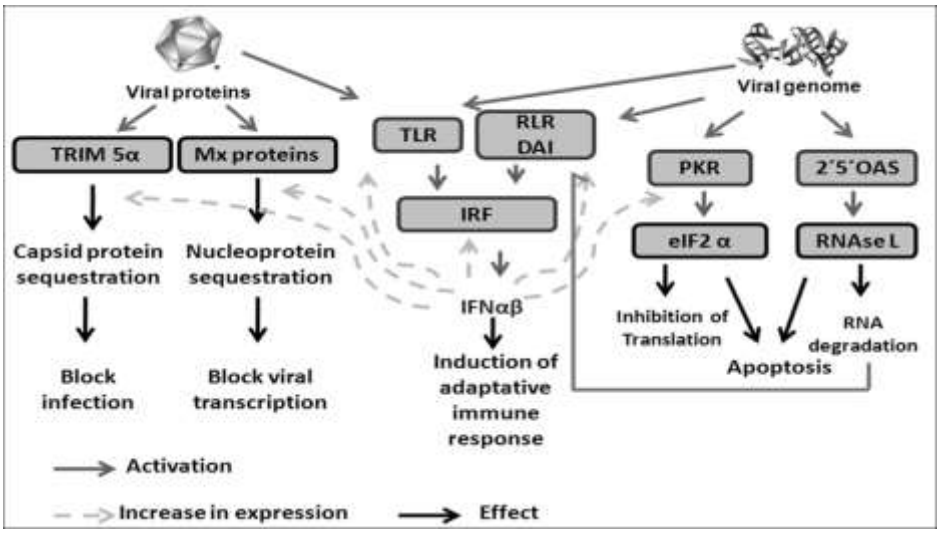

Figure 1. PRRs and antiviral proteins of the innate immune response. Viral nucleic acids and some viral proteins are recognized by TLRs, RLRs and DAI, which induce a signaling pathway that activates theproduction of cytokines and type-I IFN. IFN-mediated signaling through its cellular receptor IFNARinduces the expression of several proteins with antiviral activity. In addition, the IFN induced by virusinfection can bind to specific cell receptors and trigger the expression of different genes such as PKR. PKRis activated through homodimerisation after binding to viral dsRNAs via its dsRNA domain. Once active, as a kinase, PKR phosphorylates eIF2α (eIF2 αp) and blocks synthesis of both cellular or viral proteins. 2'5'OAS and Mx GTPase are two additional effector pathways of the IFNmediated antiviral response. Thefirst is activated by dsRNA and specifically activates the latent form of RNase L leading to RNAdegradation, and the second is activated by viral proteins and blocks viral transcription. On the otherhand, TRIM 5α recognizes the viral capsid core and blocks HIV-1 **infection at a post-entry, pre-integration stage in the viral life cycle.**

This review will focus on certain signaling PRRs that are potent triggers of inflammatory responses and play a very important role in viral infections. Among them there are the TLRs that can be either endosomal or extracellular (C. Janeway Jr & Medzhitov, 2000; Netea, van der Graaf, Van der Meer, & Kullberg, 2004), and retinoic acid-inducible gene- (RIG-) I/MDA5 (melanoma differentiation-associated gene 5), known as RNA helicase-like receptors (RLRs). Furthermore, Double-stranded RNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (2'-5'OAS), and adenosine deaminase acting on RNA (ADAR), known as effector proteins, complement the function of PRRs1. All these proteins are the main sensors of viral components and induce pro-inflammatory cytokine expression or interferon (IFN) response factors (Figure 1). This review discusses recent new knowledge on the molecular mechanism and role of PRRs in the recognition of viral PAMPs, the signaling pathways activated by such recognition, as well as the strategies used by viruses to evade such response.

TLRs, adaptor molecules and their signaling pathways

TLRs are the best characterized members of the PRR family and can recognize both intracellular and extracellular pathogens (Netea et al., 2004). Ten TLRs have been described in humans and all are type-1 membrane glycoproteins, from which six (TLR2, 3, 4 7/8, and 9) recognize viral components (Table 1). Some TLRs form hetero (TLR2/TLR1, TLR2/TLR6) or homodimers (Kang et al., 2009). TLRs have an ectodomain or extracellular domain with leucine-rich repeats (LRRs), a transmembrane region and a cytoplasmic domain, homologous to the interleukin-1 (IL-1) receptor, known as Toll/IL-1R (TIR) (C. Janeway Jr & Medzhitov, 2000). Briefly, the signaling pathway induced by TLRs is as follows: each TLR is stimulated by its specific ligand, which is recognized through the ectodomain. This leads to the recruitment, through the TIR, of adaptor proteins like the myeloid differentiation primary response protein 88 (MyD88), the Toll/IL-1 receptor domain containing adaptor protein (TIRAP), the Toll/IL-1 receptor domain containing adaptor inducing interferon-beta (TRIF), and the TRIF-related adaptor molecule (TRAM) (Figure 2).The IL-1R associated protein (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) then also participate in the signaling pathway. TRAF6 acts as an ubiquitin ligase E and activates the transforming growth factor (TGF) b-activated kinase 1 (TAK1) complex and its subunits the TAK1-binding protein-(TAB) 1 and TAB2/3, responsible for the phosphorylation of NF-kappaB essential modulator (NEMO), which results in the activation of the kinase complexes constituted by inhibitor of kappaB (IkB) and IkappaB kinase (IkK) and in the degradation of phosphorylated IkB6. Finally, the nuclear factor kB (NF-kB), the activator protein 1 (AP-1), and the IFN regulatory factors (IRFs) are released and translocated to the nucleus where regulate the expression of molecules that participate in the inflammatory response and in the initiation of innate immunity (Akira & Takeda, 2004; Netea et al., 2004) (Figure 2). The stimulation of TLR7 and TLR9 with their respective ligand results in the formation of a complex constituted by MyD88, IRAK-4, TRAF6, TRAF3, IRAK-1, IKK-k, and IRF7 responsible for the activation of IRF7, which once phosphorylated is translocated to the nucleus and regulates the expression of type-I IFN. TLR3, through TRIF as adaptor protein, can induce the phosphorylation of IRF3 translocated to the nucleus and activates NF-kB and the expression of type-I IFN, as described above (Akira & Takeda, 2004; Netea et al., 2004; Zhang & Ghosh, 2001). In summary, the stimulation of TLRs through viral PAMPs or another origin allows the recruitment of adaptor proteins responsible for activating the induction of signaling pathways that result in the activation of transcriptional factors involved in the activation of the expression of genes whose products are involved in antiviral responses.

membrane.Recognition of viral components through TLRs.

TLRs are components of a PAMP recognition system that act in concordance with other cellular factors to establish a bridge between the innate response and the adaptive immunity (Akira & Hemmi, 2003). Their activation is initiated by a signaling pathway that leads to the activation of some mitogen-activated protein kinases (MAPKs) and of transcription factors like AP-1, IRF, and NF-kB. These transcription factors are responsible for the activation of genes that encode pro-inflammatory cytokines, such as the tumor necrosis factor a (TNF-a), interleukins (IL-1, 6, 8, and 18), IFN-a/b, chemokines, leukotrienes, class II major histocompatibility molecules and co-stimulatory molecules like CD40, CD80, and CD86, necessary for adequate antigenic presentation. Initially, it was believed that the only TLRs involved in recognition of viral PAMPs were those recognizing nucleic acids like TLR3, TLR7/8, and TLR9, located in acid endosomes. However, certain authors and our unpublished results suggest that TLR2 and TLR4, located on the cell surface and known to recognize bacterial components, are also able to recognize viral PAMPs, mainly envelope glycoproteins (Compton et al., 2003; Sato, Linehan, & Iwasaki, 2006; Zhou et al., 2005). A study carried out using TLR2 and MyD88-deficient mice infected with Lymphocytic Choriomeningitis Virus (LCMV) demonstrated that the TLR2-MyD88 interaction is essential for the activation of astrocytes and microglia and for the production of TNF-a and the monocyte chemo-attractant protein-1 (MCP-1) (Zhou et al., 2005). TLR2 also recognizes PAMPs from measles virus (MV), hepatitis

C virus (HCV) and human herpes simplex virus type-1 and 2 (HSV1/2) (Bieback et al., 2002; Gaudreault, Fiola, Olivier, & Gosselin, 2007; Shehata, Abou El-Enein, & El-Sharnouby, 2006).

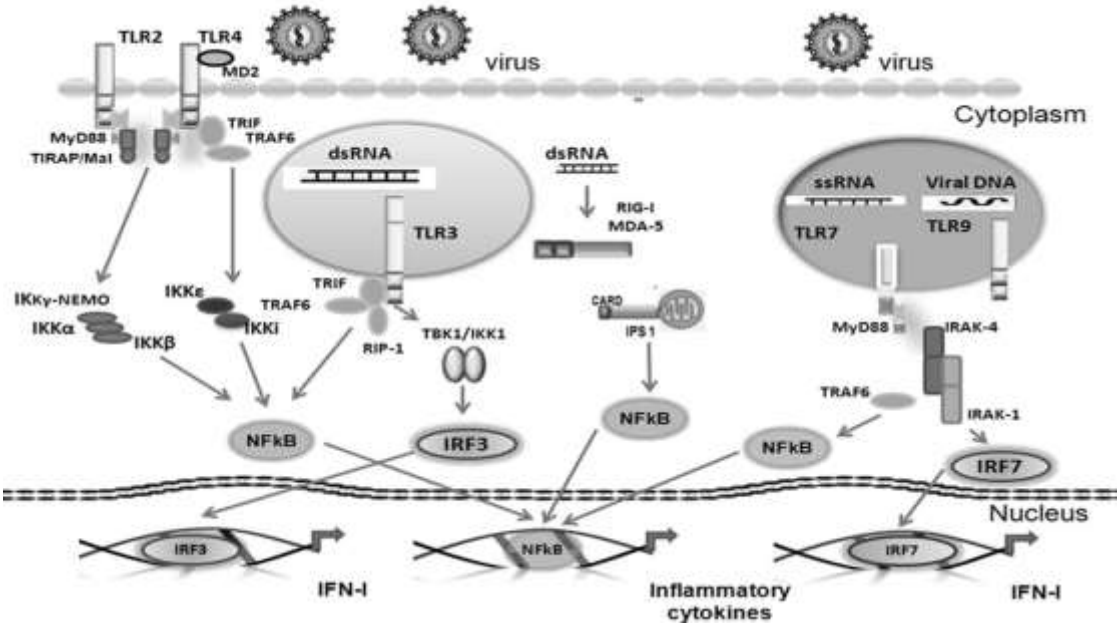

Figure 2. Viral recognition through TLRs and the RIG-I protein. TLR2 and 4 recognize mainly viral envelope proteins and recruit MyD88 by an adaptor (Mal) protein; TLR7 and TLR9 interact directly with MyD88 following ligand recognition (viral ssRNA and viral DNA, respectively**). In contrast,** TLR3 recognizes the presence of dsRNA through the recruitment of TRIF. Following MyD88 or TRIF recruitment, a signaling pathway is initiated via RIP1/TRAF6-NF-κB and TBK1/IKK-i-IRF-3/IRF-7, which ends with the expression of pro-inflammatory cytokines and type-I IFN. Recognition of dsRNA could also be mediated by RIG-I and occurs in the cytoplasm activating TBK1/IKK-i through the adapter protein IPS-1 located in the mitochondria

In the case of HCV, it was demonstrated that an increase in the level of expression of TLR2 in individuals with chronic hepatitis is related to an increase in the level of circulating TNF-a, and to the development of hepatic lesions (Shehata et al., 2006). A study performed in HEK293 cells stimulated with Epstein-Barr Virus (EBV) showed that TLR2 is stimulated through NF-kB (Gaudreault et al., 2007; J. M. Lund et al., 2004). Acute infection by varicella-zoster Virus (VZV) is characterized by the development of an inflammatory response due to high cytokine production, specifically IL-6 and IL-8, which is associated with increased expression of TLR2 in human monocytes exposed to the virus (J. P. Wang, Liu, Latz, Golenbock, Finberg, & Libraty, 2006a). TLR4 is stimulated by bacterial lipopolysaccharides (LPSs) and endogenous ligands that are inflammation products, such as fibrinogen, fibronectin, b-defensins, and heat shock proteins (Akira & Takeda, 2004). Studies performed using TLR4-deficient mice infected with vesicular stomatitis virus (VSV) showed decreased expression of IL-12 and a higher viral load in comparison to mice expressing this receptor (Haynes et al., 2001). The role of TLRs in modulating the replication of certain viruses has also been examined. Using mice transgenic for Hepatitis B virus and intravenously injected with specific ligands for TLR2, 3, 4, 5, 7, and 9 showed that all receptors, except TLR2, can inhibit virus replication in IFN-dependent manner (Isogawa, Robek, Furuichi, & Chisari, 2005). In summary, TLR4 is stimulated by PAMPs from the respiratory syncytial virus (RSV), coxsackie B virus, HSV, and MV19-21. However, additional studies must be pursued to determine the role of TLR2 and TLR4 in viral infections and in antiviral defense. All endosomal TLRs are activated by nucleic acids.

They also promote transcription of several cytokine and chemokine coding genes through the activation of NF-kB and MAPK, resulting in the expression of pro-inflammatory cytokines6. The main ligands of TLR3 are double-stranded RNA (dsRNA) genomes, and dsRNA replication intermediates of single-stranded RNA (ssRNA) viruses like RSV, dengue virus, and encephalomyocarditis virus22. TLR3 is also activated by contact with synthetic molecules or viral dsRNA analogs [poly (I:C)]. The final product of TLR3 activation is the induction of a strong inflammatory response characterized by the secretion of IL-12, TNF-a, IL-6, CXCL-10, IL-10, and IFN-a. The role of TLR3 in antiviral immune response was demonstrated in TLR3-deficient mice, which are more susceptible to murine cytomegalovirus (MCMV), given lower expression of IFN (Tabeta et al., 2004). TLR3-deficient mice infected with West Nile virus (WNV) develop higher viral loads (T. Wang et al., 2004). However, the mice are less susceptible to developing meningitis, suggesting that the entry of the virus to the brain, and its pathogenesis, could be mediated by TLR3 activation and the immune response developed upon viral infection. TLR7 is also involved in antiviral responses. In humans, TLR7 is mainly expressed by plasmacytoid dendritic cells (pDCs), which after stimulation, induce the activation of NF-kb and MAPK and trigger the expression of cytokines such as IL-6, IL-12 and IFN-a, and the activation of co-stimulatory molecules through the TLR7 pathway (J. M. Lund et al., 2004). TLR7 and TLR8 are tightly related endosomal receptors and their ligands are ssRNAs. Both receptors are also activated by guanine analogs and uridine or guanosine-rich ssRNAs of either viral or cellular origin. Both receptors are crucial for the development of adaptive immune responses during viral infections, as is the case with influenza virus and dengue virus (J. M. Lund et al., 2004; J. P. Wang, Liu, Latz, Golenbock, Finberg, & Libraty, 2006b).Finally, the endosomal TLR9 is activated by at least three types of ligands with different biological outcomes (Kumagai, Takeuchi, & Akira, 2008): i) viral DNA, ii) conventional CpG-containing DNA that activates B lymphocytes and induces the production of inflammatory cytokines by macrophages, and iii) D/A-type CpG containing-DNA that also stimulates the production of cytokines by macrophages and B lymphocytes, besides stimulating high production of type-I IFN by pDCs. It has been reported that this is the mechanism used by pDCs to respond through TLR9 to the presence of DNA viruses, such as adenoviruses, HSV1/2 and MCMV (Iacobelli-Martinez & Nemerow, 2007; Krug et al., 2004; J. Lund, Sato, Akira, Medzhitov, & Iwasaki, 2003). However, besides the recognition of DNA by TLR9, a different TLR9-independent mechanism has been proposed allowing the recognition of dsDNA in the cytoplasm of macrophages and in non-immune cells, but also inducing activation of IRF3, NF-kb, and IFN-a (Ishii & Akira, 2006).Together, this shows that upon stimulation by nucleic acids or viral proteins, TLRs induce the expression of pro-inflammatory cytokines, whose function is to protect the host from viral infection. Thus, type-I IFN is one of the main components of innate immunity and is regulated by signals initiated at both intracellular and extracellular levels.

RLRs, adaptor molecules and their signaling pathways

The RIG-I and the MAD5 are two RLRs that recognize, respectively, short dsRNAs with 5' triphosphate ends and long dsRNAs from viral genomic RNAs. Both proteins are cytoplasmic and members of the DExD/Hbox RNA helicases that are stimulated by dsRNAs. RIG-I/MDA5 have two recruiting domains: an RNA helicase domain and a caspase recruitment domain (CARD; also known as caspase recruiting domains). The first domain is responsible for the recognition of dsRNAs and for the recruitment of various proteins that activate signaling pathways, and the CARD domain recruits proteins implicated in type-I IFN expression (Kato et al., 2006). An adaptor protein has been described in this new signaling pathway. It is known as IFN-a promoter stimulator (IPS-1), [or anti-viral signaling protein (MAVS), virus-induced signaling adaptor (VISA) or CARD adaptor inducing IFN-a (Cardif)], and when it interacts with the CARD domain, allows the recruitment of dsRNA, which results in the expression of type-I IFN due to the activation and translocation of IRF3, IRF7 and NF-kB to the nucleus (Gitlin et al., 2006; Kato et al., 2005). RIG-I is up or down regulated by the ubiquitin ligase TRIM25 and by RNF125. This means that RIG-I/MDA5 recognizes viral dsRNAs in the cytoplasm, while TLR3 does so in the endosomes.

Recognition of viral components by RIG-I/MAD5: RNA helicase-like receptors (RLRs)

Certain viral dsRNAs synthesized in the cytoplasm as viral life cycle intermediaries, or as part of viral genomes are not only recognized by TLR3, but also by RLRs such as RIG-I and MAD5, which induce the expression of type-I IFN through a signaling pathway independent of the signaling activated by TLR3. Working with RIG-I- (RIG-I-/) and MAD5- (MAD5-/-) deficient mice, it was demonstrated that RIG-I is essential in recognizing RNAs from paramyxoviruses, influenza virus, and Japanese encephalitis virus; MAD5 is essential in recognizing picornavirus RNA and for the expression of IFN (Heim, 2005; Hornung et al., 2006; Yoneyama et al., 2004). These results show that RLRs recognize different types of viral RNAs and activate a signaling pathway involved in the expression of IFN, different from the pathway used by TLRs. It was recently shown that cells over-expressing RIG-I and stimulated by Newcastle Disease Virus (NDV) or VSV present increased levels of expression of type-I IFN; in contrast, cells with low-level expression of RIG-I and stimulated by NDV, VSV, Sendai virus, HCV, or WNV show decreased levels of IFN (Loo et al., 2008). Because RIG-I/MAD5a induces antiviral responses, it has been suggested that the signaling pathways of both proteins have a common adaptor protein, but it is unclear whether they act in synergy when recognizing viruses. However, MDA5 not only participates in antiviral responses, it also inhibits the growth of tumor cells, through type-I IFN (Heim, 2005; Yoneyama et al., 2004). Effector proteins involved in viral PAMP recognition. In the previous sections, the main PRRs involved in the induction of antiviral responses upon exposure to viral PAMPs were described. Nevertheless, it is also worth mentioning some proteins encoded by IFN-stimulated genes (ISGs). These proteins recognize virus structural components and through diverse mechanisms can activate antiviral responses. The first effector protein described with such characteristics was the PKR, which is an IFN-inducible serine-threonine kinase, but in contrast to PRRs, its activation does not regulate transcription, but blocks protein synthesis (Langland, Cameron, Heck, Jancovich, & Jacobs, 2006). PKR possesses two dsRNA-binding motifs (dsRBMs) in its N-terminal region and a kinase motif in its C-terminal region. As a monomer, PKR is inactive, but its binding with dsRNA or highly structured RNAs induces its homodimerization and stimulates its autophosphorylation and auto-activation (Williams, 2001). Once in the active state, PKR is dissociated from the dsRNA and phosphorylates the a-subunit of the eukaryotic translation initiation factor 2 (eIF2a), resulting in the inhibition of cellular and viral protein synthesis. PKR can also be stimulated by pro-inflammatory factors, such as growth factors, cytokines (Langland et al., 2006), or the PKR-activating protein (PACT). In virus infected cells, most of the natural PKR activators are viral dsRNAs or viral life cycle replication intermediates. However, complex viruses with DNA genomes like Vaccinia virus (VV), Adenovirus, HSV-1, or HSV-2 have open reading frames (ORFs) in opposite orientation. This allows the production of overlapping mRNA transcripts that result in the production of dsRNAs that can in turn activate PKR. Interfering RNA (RNAi) used to silence PKR, or PKR knockout mice, has shown a reduction in their expression affects IFN production upon WNV infection, making the mice more susceptible (Langland et al., 2006; Samuel, 2001). The presence of viral RNA also activates 2'-5'OAS, also known as 2'5' A synthetase. This enzyme is activated by IFN and was initially described as regulator of IFN synthesis, and, hence, as a key factor in antiviral responses (Leroy, Baise, Pire, & Desmecht, 2007; Samuel, 2001). 2´5´OAS promotes the degradation of viral RNA and catalyzes the synthesis of 2'5' oligodenylates that activate the latent cellular RNase (RNAse L) (Leroy et al., 2007). ADAR1, 2, and 3 belong to another family of proteins known as adenosine deaminase acting on RNA (ADAR). ADAR1 is induced in response to IFN and has two different isoforms: ADAR1p150 and ADAR1p110. Recently, it was described that, in infected cells, dengue virus 2 (strain TSV01) activates the expression of genes regulated by IFN, such as ADAR, PKR, and 2'5'OAS (Umareddy et al., 2008). It has also been demonstrated that ADAR1 is responsible for editing the Hepatitis delta virus RNA, independently of IFN stimulation (Hartwig et al., 2006). However, one of the most interesting aspects of ADAR is its participation in the biogenesis of microRNA, which are non-coding RNAs implicated in the regulation of the expression of genes, whose products participate in various cellular processes. Additionally, it has been reported that ADAR1-induced modifications are necessary for the maturation of such RNAs (Faller & Guo, 2008). Recently, the stimulator of interferon gene (STING)

protein, a new adaptor molecule that induces the expression of type-I IFN and plays a role in antiviral response was described. STING has 5 possible transmembrane domains; if STING is present in the endoplasmic reticulum, it can activate both NF-kb and IRF3 and induce the expression of type-I IFN (Nakhaei, Hiscott, & Lin, 2010; Zhong et al., 2008). Studies made with mouse embryonic fibroblasts that do not express STING show that such cells are susceptible to infection by viruses with negative polarity RNAs, including VSV (Ishikawa & Barber, 2008). Another mechanism of host-virus recognition is through the Fv or [tripartite motif (TRIM)] protein. Many proteins with such motifs have been described. The different isoforms are produced by alternative mRNA splicing and each variant codes for a unique domain in its C-terminus. TRIM proteins, including TRIM5a that has in its C-terminal region the B30.2 or SPRY motif, can down regulate replication of retroviruses, such as HIV-1 and Murine leukemia virus (Funderburg et al., 2008). On the other hand, some proteins members of the GTPase family, known as MX, are involved in recognition of viral proteins like the nucleocapsid, which after being recognized change their cellular location, affecting the production of new viral particles. All these mechanisms demonstrate the existence of a wide variety of strategies for pathogen recognition and host protection upon viral infections. In summary, effector proteins play antiviral roles through 4 different mechanisms: i) arrest of protein synthesis, ii) degradation of viral RNA, iii) adenosine deaminase enzymes play an important role in converting adenosines to inosines (AàI), inducing errors in translation, and iv) recognition of viral proteins. However, some of these processes can drastically affect cell viability and cause the induction of apoptosis (Umareddy et al., 2008). For this reason, the antiviral response induced by these types of proteins is immediate and observed only during the early stages of the infection, before the activation of the adaptive immune response.

Role of TLRs in viral pathogenesis

As described in this review, the aim of the recognition of viral PAMPs by PRRs is to control infection and/or contribute to the development of the adaptive immune response. However, prolonged activation of the innate immunity by TLRs can contribute to viral pathogenesis; for example, in WNV-infected mice in which the activation of TLR3 induces elevated production of TNF-a and IL-6 involved in the development of an inflammatory state, the permeabilization of the hematoencephalic barrier is induced, allowing viral entry and resulting in greater infection of the central nervous system24. Similarly, it has been described that RSV induces the over-expression of TLR3 and TLR4 that trigger an inflammatory state in the respiratory mucosa of the infected individual, who is rendered vulnerable to infections by other pathogens (Kurt-Jones et al., 2000). In cells stimulated in vitro with specific TLR2, 3, 4, 5, 7, 8, and 9 ligands and infected with HIV-1, both naïve and memory T CD4+ and T CD8+ lymphocytes are activated. T CD8+ lymphocytes are activated and begin to express CD69, which promotes their own retention in lymphoid tissues, but T CD4+ cells lose the ability to express CD69. Despite these phenotypic changes, the cell population enters the cell cycle, but grows poorly, and can even undergo apoptosis. These results suggest that the systemic activation of TLRs through diverse ligands favors immune activation, effector cell sequestering, and T cell turnover. All these events can contribute to the immune dysfunction caused by HIV-1 and to the loss of T CD4+ lymphocytes in chronic HV-1 infections (Funderburg et al., 2008).

Applications of TLRs in immunotherapies for viral disease control

Because TLRs are important for the induction of the innate immune response, understanding the molecular mechanisms involved in their activation could contribute to the development of immune therapies of vaccines, not only for viral diseases, but also for sepsis, allergies, autoimmune diseases, and cancer. TLR activation induces several different effects in immune cells, such as cytokine production, the positive regulation of co-stimulatory molecules like CD40, CD80, and CD86; for pDCs, which when stimulated through TLR9, induce IFN-a production. In this instance, besides the antiviral effect of IFN, it also influences the transport and clustering of pDCs51. This phenomenon is indispensable for the stimulation of the adaptive immune response in lymph nodes (Asselin-Paturel & Trinchieri, 2005). Other

studies also carried out with pDCs have demonstrated that early stimulation (4 h post-stimulation) with TLR7 agonists activates the antiviral cell machinery. Furthermore, stimulation of TLR7 and TLR8 with imiquimod and resiquimod, specific agonist, respectively, have therapeutic effects in basal cell carcinoma, genital lesions, and other epithelial lesions, generally associated with chronic human papillomavirus (HPV) infections (Miller, Meng, & Tomai, 2008); these compounds induce apoptosis of HPV-infected cells and of other epithelial cells with dysplasic or neoplasic changes.

Evasion of innate immunity by viruses

As a consequence of the interaction between PRRs and PAMPs, the signaling pathways activated result in cytokine and IFN expression, or in the case of effector proteins, in inhibition of protein synthesis or degradation of viral dsRNAs. However, viruses has developed strategies to evade or take advantage of such immunological barriers for productive infections. Among the strategies already described is blockage of certain steps in the synthesis of IFN-a/b, inactivation of secreted IFN molecules, interference with signaling and/or blockage of the activity of effector antiviral proteins through their sequestration, production of viral homologous proteins, or competition. In the following paragraphs, we briefly describe some of the cell components manipulated by viruses to evade the innate immune response. Expression of type-I IFN depends on the activation of IRF3 and IRF7 via IKK epsilon and TBK1. The genome of Rabies virus, Borna disease virus, and Ebola virus code for the P phosphoprotein and VP35 that can block the antiviral response induced by IFN (Brzozka, Finke, & Conzelmann, 2005; Conzelmann, 2005; Feng, Cerveny, Yan, & He, 2007). In contrast, the human herpes simplex virus 8 encodes different analogs of IRF with negative dominant activity, allowing it to interfere with the activity of cellular IRFs56. The infected cell polypeptide 0 (ICP0) from Bovine herpes virus can interact with IRF3 and induce its proteasome-dependent degradation (Saira, Zhou, & Jones, 2007). Similarly, the V protein of paramyxoviruses interacts with MD5-a and inhibits IFN-a expression (Andrejeva et al., 2004). The genome of VV encodes two proteins (A46 and A52) that specifically inhibit the TLR-dependent signaling pathway. A46 interacts with the adaptor MyD88, TRIF and TRAM; while A52 interacts with TRAF and IRAK-2, inhibiting the formation of the complex implicated in the signaling pathway (Bowie et al., 2000). During chronic infections, the NS3/4A protease of HCV degrades the TRIF adaptor of TLR3, resulting in an alteration of the antiviral response induced by dsRNA. There is also evidence regarding inhibition of PKR by viruses via several pathways: VV E3L, Influenza virus NS1, and Reovirus d3 proteins can sequester dsRNAs and prevent PKR activation. Other viruses express dsRNA or highly structured RNAs that compete for binding to PKR. EBER-1 and EBER-2, expressed during the latent state of EBV and HCV IRES are some such examples40. Inhibition of PKR can also result from protein-protein interactions, as is the case of the HCV NS5 and VV E3L that interact with PKR and inhibit its function (Feng et al., 2007; Langland et al., 2006; Samuel, 2001). The transcription factor NF-kB is essential in the induction of the IFN expression and of pro-inflammatory cytokines involved in antiviral response. However, it can also be beneficial for viruses, given that the promoters of some viruses, including HIV-1, CMV, and EBV have binding sites for NF-kb, which facilitates transcription of the viral genome. Hence, some viruses can up- or down-regulate the NF-kkb expression; for example, some proteins of the African swine fever virus are able to regulate its expression. In the early stages of infection, the early viral protein A238L inhibits NF-kb expression, but once the infection progresses the late viral protein A224L stimulates its expression (Tait, Reid, Greaves, Wileman, & Powell, 2000). Furthermore, besides the direct strategies inhibiting IFN expression, VV expresses the A52R protein, which contains a TIR domain that interacts with the cytoplasmic proteins IRAK-2 and TRAF6 involved in TLR

HIV-1 infection at a post-entry, pre-integration stage in the viral life cycle.

compartment as described for the PAMPs of other pathogenic agents. From this perspective, compartmentalization of the innate system has some similarity with the adaptive immune system because both systems use certain factors, depending on their cellular location. It is clear; however, as shown here,

that upon infection, the immune response is initiated with the recognition of viral PAMPs by PRRs, resulting in the up-regulation of IFN-a/b. Since the discovery of IFN, 50 years ago, knowledge about the signaling pathways that regulate its production has greatly expanded. The discovery of different PRRs has led to better understanding the interactions between the innate and adaptive immune responses and has favored the development of new therapies owing to the discovery that the stimulation of TLRs plays an important role in the protection from, or in the development of diseases. This highlights the importance of studies aiming to better understand the regulatory mechanisms and the signaling pathways associated with the PRRs in response to PAMPs.Other molecules that have also gained importance are the effector proteins, which inhibit protein synthesis, degrade RNA, or modify viral genomes and are also involved in launching the activation of the innate immune system and in developing the adaptive immune response.

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Chapter 3

Scope of the thesis

The work described in this thesis focuses on the early events associated with dengue virus (DENV) infection. DENV is transmitted to humans via mosquito bites and targets cells of the host immune system including monocytes/macrophages and dendritic cells (DCs) (Kurane & Ennis, 1988; Marovich et al., 2001; Wu et al., 2000). There are 5 distinct DENV serotypes (ScienceInsider, 2013) and antibodies generated during a primary infection appear to have a dual role in controlling DENV re-infection. Upon re-infection, the pre-existing antibodies bind to the newly infecting virus and target the virus to Fc-receptor (FcR) expressing cells, including monocytes/macrophages and DCs (Boonnak et al., 2008; Halstead & O'Rourke, 1977a; Halstead, 2003; Kliks et al.,1989). Viral entry is subsequently facilitated by antibody-FcR interaction (Boonnak et al., 2013). At high antibody titers, viral infectivity is neutralized and no specific disease symptoms develop. At sub- or non-neutralizing antibody concentrations, however, enhanced DENV infectivity is seen in monocytes, macrophages and DCs indicating that DENV can escape from degradation (Boonnak et al., 2008; Halstead & O'Rourke, 1977a). This phenomenon - also known as antibody-dependent enhancement (ADE) of infection– is associated with the development of severe disease (Burke & Kliks, 2006; Halstead & Simasthien, 1970; Halstead, 2003). Severe disease is predominantly seen in individuals with a heterologous secondary DENV infection and during primary DENV infection of infants born to dengue-immune mothers (Burke & Kliks, 2006; Halstead & Simasthien, 1970; Halstead & O'Rourke, 1977b; Simmons et al., 2007). Thus, during primary as well as secondary infection, the cells that are the primary sentinels of host immunity become viral factories. My objective was to unravel if and how DENV infection with and without antibodies influences the function of these cells, with emphasizes on DCs. To answer this, we characterized the activation status of cells targeted by DENV in ex-vivo and in vitro models. Additionally, we investigated the infectious potential of immature dengue virions and corroborated their importance in severe disease pathogenesis.

Chapter 4 provides an ex-vivo analysis of monocytes and DCs isolated from dengue-infected patients in the acute and convalescent phase of the disease. Several in vitro and ex vivo studies revealed that Toll-like receptor (TLR) signaling is altered by DENV infection (Azeredo et al., 2010; de Kruif et al., 2008; Modhiran, et al., 2010; Sun et al., 2009). Therefore, we here analyzed the expression of several TLRs and co-stimulatory molecules on monocytes and DCs from patients with different clinical outcomes. We found a link between lower expression of TLR3 and TLR9 and disease severity. In addition, DCs of severe dengue cases were observed to express lower levels of CD80 and CD86. Lastly, we demonstrate that interferon alpha production decreases in the presence of dengue virus after stimulation of PBMCs with TLR9 agonist.

In Chapter 5, we evaluated if the presence of waning titers of anti-DENV-2 specific serum influences DENV-2 infection and activation of immDCs. In contrast to what has been previously described (Dejnirattisai et al., 2011; Nightingale et al., 2008), we found that only a small percentage of immDCs supports DENV infection. The vast majority of immDCs do internalize virus particles but apparently DENV fails to achieve a productive infection in most cells. Furthermore, in line with a previous study (Boonnak et al., 2008), waning antibody titers do not enhance DENV infection in immDCs. Also, we show that while infection in the absence of antibodies impairs DCs activation, DCs acquire a fully mature state following infection at high antibody titers. Notably, infection of DCs at non-neutralizing serum conditions was found to trigger an intermediate, semi-mature phenotype.

ImmDCs proved to be relatively permissive to DENV infection in the absence of antibodies and fail to support ADE (Chapter 5, refs). On the other hand however, mature DCs are believed to be low/non permissive to DENV infection but do support enhanced infection in the presence of non-neutralizing antibodies (Boonnak et al., 2008). Therefore, in chapter 6, we evaluated the relative role of immature and mature DCs in DENV-2 infection in the absence and the presence of non-neutralizing antibodies. We investigated the specific infectivities of viruses produced in these two DC populations and show a striking difference in the quality of the viral progenies.

DENV-infected cells secret virions that vary in maturation state from fully mature to fully immature particles (Cherrier et al., 2009; Junjhon et al., 2010). The latter are considered non-infectious, however their infectivity can be rescued by DENV-specific antibodies (Dejnirattisai et al., 2010; Yu et al., 2008; Zybert et al., 2008). Interestingly, partially immature particles of West Nile Virus (WNV) – like DENV a member of the flavivirus genus – was shown to infect cells expressing DC-SIGN (Mukherjee et al., 2011). The glycan moieties on prM were found to interact with DC-SIGN, thereby facilitating virus binding and cell entry. Therefore in chapter 7, we revisited the infectious properties of immature DENV and tested its ability to infect immature DCs. We show that immature DENV exhibits low infectivity in immature DC, which is strictly dependent on its interaction with DC-SIGN receptor. Additionally, we demonstrate that, consistent with data on standard DENV preparations, macrophages but not immature DCs support ADE of immature virus infection.

In chapter 8 we investigated the contribution of immature DENV particles in severe disease. We analyzed the capacity of acute DENV-2 sera from patients with DF, DHF and DSS to bind and enhance infection of immature DENV-2. We conclude that immature particles and antibodies recognizing these virions act as a co-factor in disease pathogenesis.

In chapter 9 and 10, I summarize and discuss the key findings of the thesis.

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Part II

Dendritic cells in DENV infection: From sentinels of immunity to viral targets

Chapter 4

Differential Expression of Toll-like Receptors in Dendritic Cells from Patients with Dengue during Early and Late acute phases of the disease

Silvia Torres, Juan Carlos Hernández, Diana Giraldo, Margarita Arboleda, Mauricio Rojas, Jolanda M Smit, and Silvio Urcuqui-Inchima

Dengue hemorrhagic fever (DHF) is observed in individuals that have pre-existing heterotypic dengueantibodies and is associated with increased viral load and high levels of pro-inflammatory cytokines early in infection.Interestingly, a recent study showed that dengue virus infection in the presence of antibodies resulted in poor stimulation of Toll-like receptors (TLRs), thereby facilitating virus particle production, and also suggesting that TLRs may contribute to disease pathogenesis.We evaluated the expression levels of TLR2, 3, 4 and 9 and the co-stimulatory molecules CD80 and CD86 by flow cytometry. This was evaluated in monocytes, in myeloid and plasmacytoid dendritic cells (mDCsand pDCs) from 30 dengue patients with different clinical outcomes and in 20 healthy controls. Increased expression of TLR3 and TLR9 in DCs of patients with dengue fever (DF) early in infection was detected. In DCs from patients with severe manifestations, poor stimulation of TLR3 and TLR9 was observed. In addition, we found a lower expression of TLR2 in patients with DF compared to DHF. Expression levels of TLR4 were not affected. Furthermore, the expression of CD80 andCD86 was altered in mDCs and CD86 in pDCs of severe dengue cases. We show that interferon alpha production decreased in the presence of dengue virus after stimulation of PBMCs with the TLR9 agonist (CpG A). This suggests that the virus can affect the interferon response through this signaling pathway.These results show that during dengue disease progression, the expression profile of TLRschanges depending on the severity of the disease. Changes in TLRs expression could play a central role in DC activation,thereby influencing the innate immune response

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Author summary:

Dengue is a public health problem worldwide and the most prevalent mosquito-borne viral disease affecting humans. Dengue virus (DENV) infections causes a broad spectrum of clinical manifestations, ranging from self-limited fever to severe disease, such as dengue hemorrhagic fever (DHF), that can be fatal. The pathogenesis of severe dengue is associated with an inadequate immune response characterized by the over-production of cytokines and other inflammatory components. However, little is known about the role of the innate immune response in the progression to hemorrhagic manifestations. TLRs constitute one of the most important components of innate immunity and are responsible for initiating a response against a variety of pathogens, including viruses. Recent studies suggest that TLRs may contribute to disease pathogenesis. Here we aimed to explore the role of these receptors in dengue disease progression. To this end, we examined the expression of several TLRs and of co-stimulatory molecules in monocytes and DCs from dengue patients. A link between TLRs expression and severity of dengue was observed: patients with dengue fever express higher levels of TLR3 and TLR9 than patients with DHF. This could be crucial in host defense against dengue virus or disease progression. In addition, expression of CD80 and CD86 was altered in DCs of severe dengue cases. We show that interferon type I production is also altered in vitro through TLR9. This suggests that dengue virus affects the interferon response through this signaling pathway.

Introduction

Dengue is the most widespread mosquito-borne viral disease worldwide. It is estimated that 50 million dengue infections occur each year, and that 2.5 billion people are at risk of acquiring dengue virus (DENV) infection (WHO, (2009)). It is caused by any of the four distinct, but closely related DENV serotypes (DENV-1 to 4), that are members of the Flaviviridae family (Lindenbach & Rice, 2003). DENV infection may lead to a febrile illness known as dengue fever (DF) but can also result in life-threatening complications defined as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (WHO, 1997).

Clinical and epidemiological studies have revealed that a secondary infection with a heterotypic serotype is a risk factor for the development of severe dengue disease (de Kruif et al., 2008; Halstead & Simasthien, 1970). Furthermore, infants born from dengue-immune mothers are at risk of acquiring severe dengue disease during a primary infection (Chau et al., 2008). The development of severe disease has therefore been linked to the presence of pre-existing antibodies. Although the mechanisms involved in immune-enhanced disease are still poorly understood, multiple in vitro studies have shown that antibodies promote viral entry and suppress antiviral responses, allowing a higher production of virus particles per infected cell (Boonnak, Dambach, Donofrio, Tassaneetrithep, & Marovich, 2011; van der Schaar, Wilschut, & Smit, 2009; Vaughn et al., 2000). The high infected cell mass and viral load seen early in infection together with an aberrant T cell response is believed to induce a cytokine storm which causes the hemorrhagic manifestations.

The first line of defense towards pathogens is mediated by the innate immune system. Key players in the initiation of the innate immune response are Toll-like receptors (TLRs). TLRs recognize invaders through detection of pathogen-associated molecular patterns. To date, 10 TLRs have been described in humans, 6 of which (TLR2, 3, 4, 7, 8, and 9) are implicated in recognition of viral components; namely viral nucleic acids and proteins (Takeda & Akira, 2007). TLRs are abundantly expressed on monocytes, macrophages and dendritic cells (DCs) (Jarrossay, Napolitani, Colonna, Sallusto, & Lanzavecchia, 2001; Zarember & Godowski, 2002), the main target cells of DENV, and trigger antiviral defenses such as the production of interferon and pro-inflammatory cytokines. Activation of TLR3 and TLR7 inhibits DENV replication in the monocyte cell line U937 and the human cell line HEK293, respectively (Tsai, Chang, Lee, & Kao, 2009), indicating that these TLRs possess antiviral activity towards DENV. Intriguingly, however, when DENV cell entry is facilitated by antibodies, activation of TLR-negative regulators and down-regulation of TLR4 and genes associated with TLR signaling, have been observed in the monocyte cell line THP1 (Modhiran, Kalayanarooj, & Ubol, 2010). These results indicate that immune suppressive mechanisms are activated through this mode of viral entry. Similar results were found in peripheral blood mononuclear cells (PBMCs) from patients experiencing secondary DHF but not DF. Furthermore, and in line with the above observations, several clinical studies have indicated that alterations in pro-inflammatory cytokine production, as observed in DHF patients, can be attributed to recognition through TLRs (). Taken together, the recognition and subsequent activation of TLRs may be a contributing factor in dengue disease pathogenesis. In this study we examined the expression level of TLR2, 3, 4 and 9 and of the co-stimulatory molecules CD80 and CD86 (CD80/CD86) in dengue patients experiencing distinct disease manifestations. TLRs and CD80/CD86 expression was evaluated in the acute and convalescent phase of the disease. The expression patterns of the different TLRs were assessed in each cell population as follows: monocytes (TLR2/4/9) (Hornung et al., 2006; Nguyen et al., 2010), plasmacytoid DCs (pDCs; TLR2/9) (Hemmi et al., 2002; Hernandez, Arteaga, Paul, Kumar, Latz, & Urcuqui-Inchima, 2011a; Hernandez, Arteaga, Paul, Kumar, Latz, & Urcuqui-Inchima, 2011b) and myeloid DCs (mDCs; TLR2, 3, 4 and 9) (Kadowaki et al., 2001; Nguyen et al., 2010; Perrot et al., 2010). These cells represent important targets of DENV infection [22], and are key players in the innate immune response (Cella, Sallusto, & Lanzavecchia, 1997). Our data indicates there is a differential regulation of TLR expression profiles during the acute phase of DF and DHF disease.

Materials and methods

Ethics statement

The protocols for patient enrollment and sample collection were approved by the Committee of Bioethics Research of the Sede de Investigación Universitaria, Universidad de Antioquia (Medellín, Colombia), as well as by the informed consent form, according to the principles expressed in the Declaration of Helsinki. All subjects read and signed an informed consent (including healthy donors). When the participant was a minor, the informed consent was signed by at least one parent.

Study populations and blood samples

Thirty DENV-infected patients, 13 female and 17 male subjects between 12-72 years of age were enrolled in this study. Twenty healthy, 10 females and 10 males 13-52 years old, were included as healthy controls (HC). All HC were negative for DENV NS1 antigen and DENV IgM/IgG and have not been vaccinated against yellow fever virus. Dengue patients were enrolled from May 2009 to February 2010 in five healthcare centers located in Turbo and Apartadó, two municipalities of Antioquia, Colombia.
Thirty ml of peripheral blood (PB) were taken and added to EDTA-containing tubes. Blood samples were collected three times, on the 3rd and 5th day after the beginning of symptoms (acute samples) and 15 days after admission to the hospital (convalescence samples). Infection with dengue was confirmed if one of the following tests were positive (1) Platelia™ Dengue NS1 Antigen kit (Bio-Rad Laboratories, Marnes La Coquette, France), (2) DENV specific real-time RT-PCR (Chutinimitkul, Payungporn, Theamboonlers, & Poovorawan, 2005), (3) DENV IgM detection by ELISA UMELISA®.

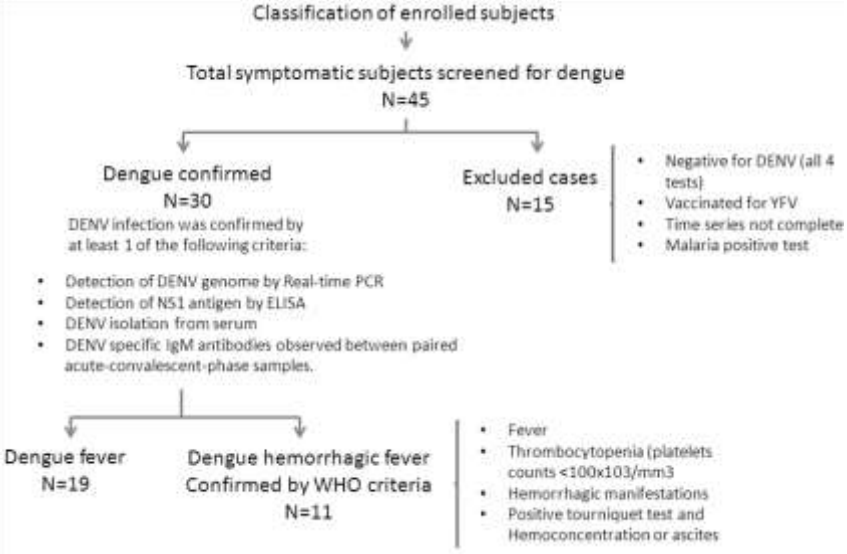


Figure 1. Flowchart of enrolment, inclusion/exclusion criteria, diagnosis and classification of dengue patients.

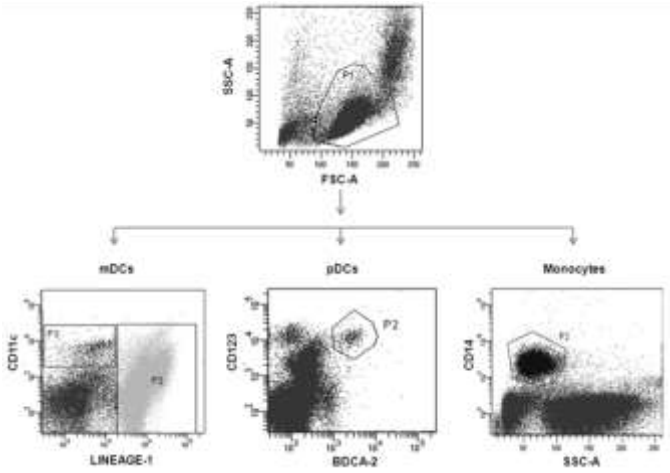
Inmunoensayo, Instituto Pedro Kourí, La Habana, Cuba) or (4) virus isolation and propagation in C6/36 mosquito cells (Singh & Paul, 1969). To determine whether the patient had a primary or secondary infection, the presence of dengue-specific IgM/IgG antibodies was evaluated, using the PanBio Dengue Duo Cassette System (PanBio Ltd, Queensland, Australia). Anti-dengue IgG levels were determined on day 3, on day 5 and on day 15 after the beginning of symptoms and if there was no rise in IgG titer over time it was classified as secondary infection. Dengue cases where classified as DF or DHF according to the guidelines of the World Health Organization (WHO) of 1997 (WHO, 1997), we applied the old guidelines as the new WHO guidelines published in 2009. The new guidelines are more focused on clinical practice and are not widely accepted for use in research (Srikiatkhachorn et al., 2011). Clinical characterization of DHF included the following criteria: fever, thrombocytopenia (platelet counts <100x103/mm3), hemorrhagic manifestations, positive tourniquet test, and hemoconcentration (20% changes in hematocrit value) or ascites as evidenced by plasma leakage. A flow chart explaining the in/exclusion criteria is depicted in figure 1.

Flow cytometry

PB samples were used to determine the frequency of mDCs, pDCs and monocytes and their expression of TLRs. All patients and healthy controls the proportion of mDCs, pDCs, and monocytes among blood mononuclear cells were 0.5% (0.4-1.6%) 0.3% (0.1-1%), and 6% (4-12%), respectively. Peripheral blood was incubated with the appropriate antibodies at room temperature for 25 minutes. The whole blood was lysed with lysing Buffer BD Biosciences, San Jose, CA during 10 minutes at room temperature. Leucocytes were resuspended and washed in PBS containing 0.5%

BSA and 0.1% sodium, fixed with 2% formaldehyde and stored at 4ºC until analysis. For staining of intracellular receptors (TLR3 and TLR9), the cells were treated with fixation permeabilization buffer from (eBiosciences, San Diego. CA) following the manufacturer's recommendations. All samples were evaluated within 2-4 h of staining and 200,000 events were acquired per tube. Analyses were performed using the BD
FACS Dive V6.1.1 (BD Biosciences), and the operating software on the FACS Canto II flow cytometer. TLR levels were expressed as mean fluorescent intensity (MFI) compared to the isotype-control. Logical gating was used to identify mDC, pDC and monocyte populations. The evaluation was done by single tube analysis. The acquisition gate (P1) was common for all the populations and was established based on forward scatter (FSC) and side scatter (SSC) corresponding to the gate of mononuclear cells (approximately 130,000-170,000 events). For phenotyping of each sub-population the following strategy was used: mDCs (Lin1-/CD11chigh) were gated as P3, Lin1 positive cells were excluded from analysis (gate P2), the pDCs (BDCA-2+/CD123high) were gated as P2 and monocytes (CD14+) were gated as P2 (Fig. 2A). Each specific sub-population was plotted as a histogram to show the expression of TLR2, 3, 4 and 9 (Fig. 2B). The data are presented as overall mean fluorescence intensity (MFI) for toll-like receptors (TLRs) on the TLR+ subset, after subtraction of isotype staining background. The following monoclonal antibodies were used: Lineage 1 FITC (anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20, and anti-CD56 cocktail), anti-CD123 PE-Cy5, anti-CD11c PE-Cy5, anti-CD80 PE and anti-CD86 PE (BD Biosciences, San Jose, CA). Anti-TLR2, 3, 4 and 9 were PE conjugates (eBiosciences, San Diego, CA). Anti-BDCA-2 FITC was from (Miltenyi Biotec, Auburn CA). Unstained cells and conjugated isotype antibodies were used as controls; all of them matched for concentration with the primary antibodies. All reagents were used according to the manufacturer's instructions

A



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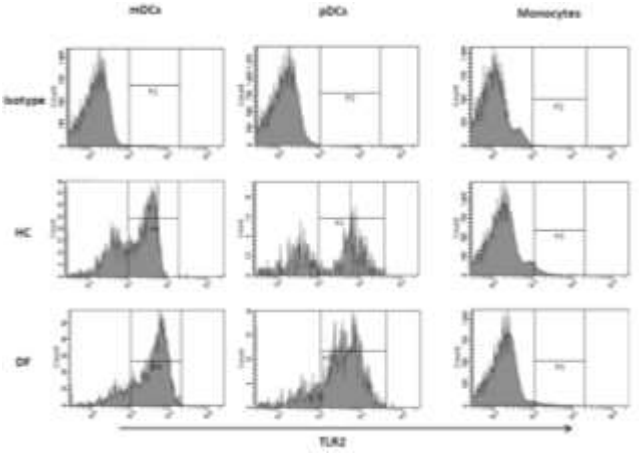

Figure 2. Gate strategy for identification of mDCs, pDCs and monocytes from PB samples and TLR staining. (A) Non duplets population was fractioned in mDCs as mononuclear cells Lin^- and $CD11c^{High}$ (P3); (pDCs) as $CD123^+$ $BDCA2^+$ (P2) and monocytes as $CD14^+$ (P2) . (B) Representative examples of TLR2 expression in mDCs, pDCs, and monocytes. HC indicates healthy control and DF denotes for dengue fever.

Virus stocks and titration

The DENV-2 New Guinea C strain (NGC) was provided by the Center for Disease Control (CDC, Fort Collins, CO), and propagated in the C6/36 mosquito cell line. C6/36 cells were grown in L15 medium (Invitrogen, Carlsbad. CA) supplemented with 10% heat-inactivated fetal bovine serum, 1% vitamins, 1% L-glutamine and 1% non-essential amino acids (Sigma-Aldrich Chemical Co, St. Louis, Mo), and incubated at 34°C without CO2 for 24 h. The cells were inoculated with DENV-2 at 0.01 multiplicity of infection (MOI) and incubated at 34°C for 5-6 days. The supernatants were collected and clarified by centrifugation (1500 g, 10 min). The virus titer was determined by conventional plaque assay in kidney rhesus monkey cells, (LLC-MK2), essentially as described (Srikiatkhachorn et al., 2011). Inactivation of the virus was achieved by exposure to UV light for 60 min.Influenza A virus (IAV) strain A/PR8/34 was kindly donated by Paula Velilla (Immunology Group, Universidad de Antioquia). Virus titration was performed by the limit dilution method, using 96-well microplates (Nunclon, NY). The virus titer was estimated by the cytopathogenicity of the cells and expressed as 50% tissue culture infectious doses/ml (TCID50/ml) of IAV ; 1x104 TCID50/ml were used for the infection of PBMCs. The supernatant from IAV-infected PBMCs was used as a positive control to measure interferon concentration by ELISA (eBioscience, San Diego. CA).

Preparation and infection of PBMCs

PBMCs were isolated from HC by density gradient centrifugation using Ficoll-Hypaque (Histopaque 1077, Sigma Aldrich Chemical Co., St. Louis, MO); all samples were processed within the first 8 h after collection. PBMCs were cultured at 1.0x106 cells/ml in 24-well polystyrene tissue culture plates at 37ºC in 5% CO2, using RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin/streptomycin (Sigma Aldrich Chemical Co., St. Louis, MO) and 1% of L-glutamine (Sigma Aldrich Chemical Co., St. Louis, MO). Subsequently, the cells were challenged with wild-type DENV or UV-inactivated (iDENV), at a MOI of 5, for 2 h at 37°C in 5% CO2. The PBMCs were then washed and incubation was continued for another 24 h in the presence or absence of a TLR agonist: 50 µg/ml of polyinosinic:polycytidylic acid [poly(I:C)], 10 µg/ml of lipopolysaccharide (LPS), 5 µg/ml resiquimod (R848) and 5 µM oligonucleotides with motifs of unmethylated cytosine-phosphate-guanine (CpG) dinucleotides type A (CpG A); (Invivogen .San Diego. CA), for TLR3/TLR4/TLR7/8 and TLR9, respectively. To neutralize the stimulatory effect of CpG ODNs, 5 mM ODN TTAGGG was used (Invivogen .San Diego. CA). The supernatants were harvested after 24 h of culture and the interferon alpha (IFN-α) concentration was measured by ELISA according to the manufacturer's protocol (eBioscience, San Diego. CA).

Statistical analyses

Statistical comparisons between the healthy donors and the dengue groups were performed using the Kruskal-Wallis test, with a confidence level of 95% followed by Dunn's multiple comparison test. To compare TLRs expression according the severity of the disease, a Mann-Whitney test was employed. The critical value for statistical significance used for the analyses was $P<0.05$. All the analyses were carried out using the GraphPad prisma (Graph Pad, CA) software.

Results

Characteristics of patients

In this study, 45 patients were enrolled, 30 of which were positive for DENV infection. From the 30 dengue positive cases, 19 (67%) were classified as DF- and 11 as DHFpatients (Fig. 1), based on the guidelines of the WHO (WHO, 1997). The patients were admitted to the hospital and PB samples were taken on days 3, 5, and 15 after onset of symptoms. Six patients (26.6%) developed ascites related to dengue infection; there were no patients with DSS nor mortal cases. The demographic and clinical information of the 30 dengue patients enrolled in this study are summarized in Table 1

Demographic and clinical characteristics of 30 individuals with diagnosis of dengue and 20 healthy controls

	Controls	**DF***	**DHF***
	n= 20	n= 19	n=11
Age, yr, mean ±SD	32.61(16.41)	30.94(18.24)	33.09(12.56)
Gender			
Male	10	11	6
Female	10	8	5
Severity criteria, no. (%)			
Thrombocytopenia*		3(15.78)	11(100)
Hemoconcentration		8(42.10)	9(81.81)
Ascites		2(10.52)	4(36.36)
Spontaneous hemorrhage		4(21.05)	8(72.72)
Positive tourniquet result		5(26.31)	9(81.81)

Units specified in parenthesis are percentages; otherwise data are numbers
(DF)*=Dengue fever; (DHF)*=Dengue Hemorrhagic fever.
Thrombocytopenia* = Platelet counts <100000/mm3.
Hemoconcentration*= Hematocrit level rising $\geq$ 20%
Ascites*=accumulation of fluid in the peritoneal cavity confirmed by abdominal ultrasound.
Spontaneous hemorrhage=nose bleeding, gastrointestinal bleeding, ocular bleeding, and/or bleeding gums.
Positive tourniquet= petechiae of $\geq$20 spots in a 2.5-cm^2 area on the forearm after application of pressure at the midpoint between systolic and diastolic pressure for 5 min using a sphygmomanometer.

TLR expression is up-regulated in mDCs and pDCs during the acute phase of dengue

DCs are not only important target cells of DENV infection; they also play a central role in the innate antiviral response, through TLRs activation. Therefore, we evaluated the expression levels of TLR2, 3, 4 and 9 in mDCs, pDCs and monocytes of dengue patients, and compared them to HC. In mDCs of dengue patients, TLR3, TLR4 and TLR9 reached maximum expression on day 5 of illness (Fig.3A).
The MFI results showed that expression of TLR3 in mDCs was significantly higher in dengue patients than in HC, on days 3 and 5 of illness ($P<0.05$ and $P<0.01$, respectively).(Fig. 3A). For TLR4, only at day 5 a significantly higher expression was observed (Figure 3A),. Expression of TLR9 in mDCs of dengue patients was significantly increased ($P<0.05$) on day 5 of illness compared to those of HC, while expression of TLR2 in mDCs did not differ between dengue patients and HC (Fig. 3A). Our results also show that on day 15, the level of expression of all the TLRs tested decreased to levels similar to those of HC (Fig 3A).
Unlike in mDCs, in pDCs of dengue patients, only TLR2 expression was stimulated. The MFI of TLR2 reached maximum peaks at day 5 of illness. (Fig. 3B). On day 15, TLR2 expression was similar to that of HC (Fig. 3B),

suggesting that after DENV infection, TLR2 expression tends to normalize. In pDCs, the expression of TLR3, 4 and, 9 did not differ between dengue patients and HC (Fig. 3B). In monocytes, no differences in TLR expression levels were detected between dengue patients and HC (data not shown). Similar results were obtained when the TLRs frequency was evaluated on the basis of absolute cell counts (data not shown).

Differential expression of TLR2 in mDCs and pDCs of DF and DHF patients during the acute phase of infection

The development of DHF is associated with an increased level of circulating pro-inflammatory cytokines and chemokines (Chang & Shaio, 1994; Gagnon et al., 2002; Lee et al., 2006), and several in vitro studies indicate that TLRs may contribute to this phenomenon (Azeredo et al., 2010; de Kruif et al., 2008). Therefore, we compared the expression profile of TLR2 and TLR4 in mDCs, pDCs and monocytes of patients with DF and DHF on different days of disease to that of HC. Similar TLR2 and TLR4 expression levels were detected in monocytes of DF, DHF patients and HC (data not shown). In contrast, there was a modulation in TLR2 expression in DCs of DF and DHF patients. On day 3 of illness, DF patients presented significantly lower TLR2 expression in mDCs, compared to HC and DHF ($P<0.05$; Fig. 4A). On days 5 and 15, the level of TLR2 expression was similar in patients with DF and DHF. In pDCs of DHF patients, a significant increase in TLR2 expression was seen on day 3 of illness, compared to HC ($P<0.05$; Fig. 4B). This increase reached a maximum on day 5 of acute infection ($P<0.001$). No differences were found in TLR4 expression levels in mDCs (data not shown).

TLR3 and TLR9 expression is higher in DCs of DF than in those of DHF patients

The effect of DENV on intracellular TLRs expression was evaluated in cell lines and in animal models (Nasirudeen et al., 2011; Wang et al., 2006). However, the effect of DENV infection on the modulation of intracellular TLRs expression in DCs of patients with DF or DHF has not been reported. The results show that on day 5 after the beginning of symptoms, mDCs of DF patients had a higher MFI for TLR3 and TLR9 than the mDCs of DHF patients ($P<0.05$) and HC ($P<0.001$

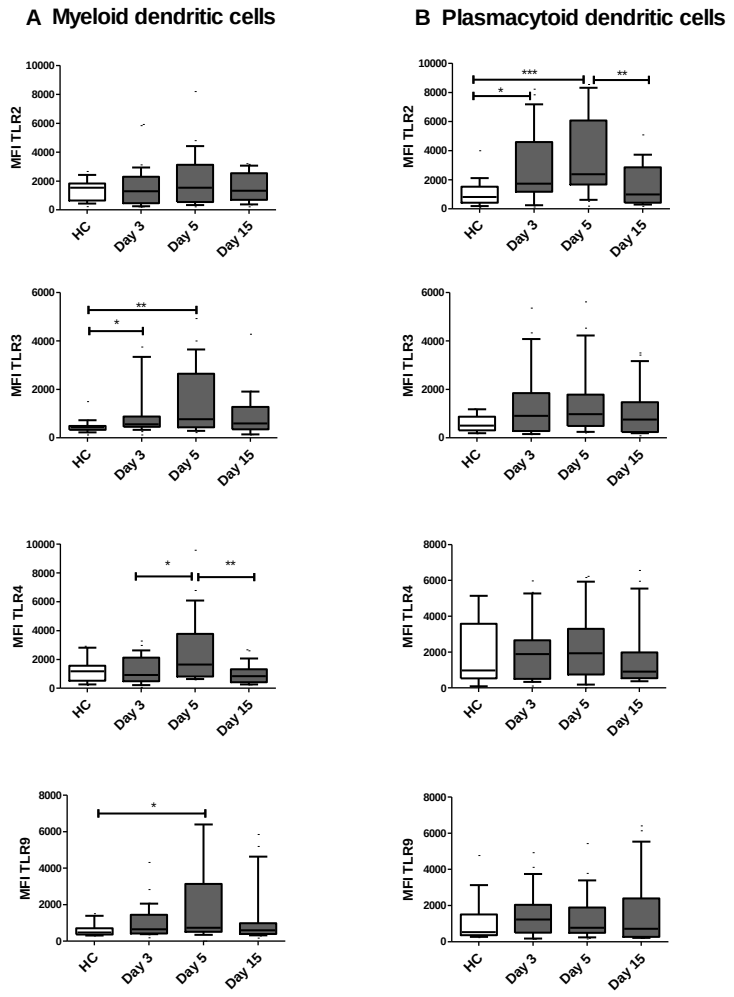

Figure 3. Increased expression of TLRs in mDCs and pDCs of dengue-infected patients. Expression profile of TLR2, 3, 4 and 9 in mDCs and pDCs of dengue patients isolated at different times after the onset of disease symptoms (day 3, 5 and 15) (n = 30) and of healthy controls (n = 20). The evaluation of TLR expression was performed by flow cytometry based on mean fluorescence intensity (FMI) analysis. Data are presented as the median and 25–75 interquartile ranges Statistical analysis was performed by using the Kruskal-Wallis test followed by the Dunn's Multiple comparisons Test. *p< 0.05, ** p< 0.01 and ***p< 0.001

and P<0.01), respectively; Fig. 5A and B). On day 3 of illness, mDCs from DF or DHF showed a higher MFI for TLR3 and TLR9 compared to that of HC (Fig. -5A). In the

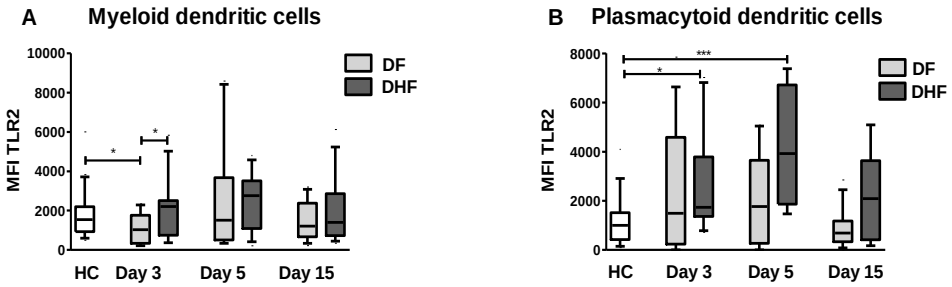

Figure 4. Increased TLR2 expression in pDCs of patients with severe disease. Analysis of TLR2 expression by flow cytometry of PB mDCs and pDCs cells in DF patients (n= 19) and DHF patients (n=11) at different times after the onset of symptoms (days 3, 5 and 15). Data are presented as the median and 25–75 interquartile ranges. Statistical analysis was performed by the Mann Withney test.*$p< 0.05$, ** $p< 0.01$ and ***$p< 0.001$.

convalescent phase, the expression levels of TLR3 and TLR9 decreased to similar levels as in HC. The MFI results for TLR9 in pDCs of DF patients showed higher expression levels on day 3 of illness compared to those of DHF patients (P<0.05; Fig. 5A). Taken together, our results are consistent with recently published in vitro data (Nasirudeen et al., 2011; Tsai et al., 2009; Wang et al., 2006), and suggest that the increase in TLR3 and TLR9 expression in DF patients could act as an antiviral factor.

Down-modulation of CD80/CD86 in mDCs of DHF patients

Infection of mDCs with DENV has been reported to induce DC maturation and activation, albeit to lower levels than observed in uninfected DCs (Libraty, Pichyangkul, Ajariyakhajorn, Endy, & Ennis, 2001; Palmer et al., 2005). Since the effect of DENV on DC maturation is unclear in vivo we here assessed the maturation state of the mDCs and pDCs by examining the expression profile of the co-stimulatory molecules CD80 and CD86 in DF and DHF patients.Because the co-stimulatory molecule CD80 appears to be expressed in resting unstimulated mDCs (Velilla et al., 2008), we quantified the expression level of CD80 in mDCs of patients with DF and DHF and compared it with the expression in mDCs of HC. Analysis of the MFI showed that the mDCs of DHF patients express significantly lower levels of CD80 (P<0.05) and CD86 (P<0.001), when compared to HC (Fig. 6A and 6B). Also in pDCs, a significant decrease in the expression level of CD86 was seen in patients with DF and DHF (P<0.05 and P<0.01, respectively), compared to HC. No differences were found in the expression of CD80 in pDCs (data not shown) Taken together, we observed a low expression level of TLR3 and TLR9 and of CD80 and CD86 in DCs of DHF patients.

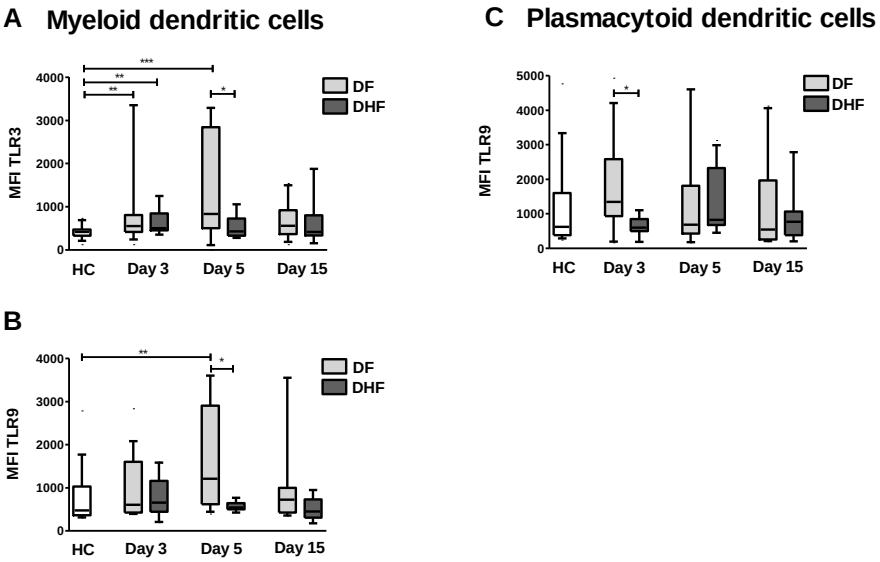

Figure 5. TLR3/TLR9 in mDCs and TLR9 in pDCs have different expression levels depending on disease severity. Analysis of TLR expression levels was performed as described in the legend of Figure 3.

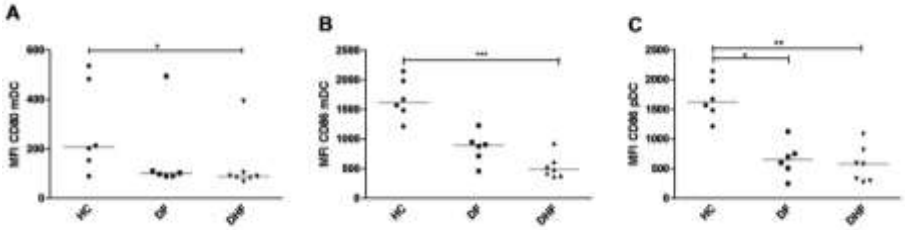

Figure 6. Expression of the co-stimulatory molecules CD80 and CD86 is affected in mDCs of DHF patients. Expression of CD80 and CD86 was evaluated by flow cytometry based on the FMIanalysis in mDCs and pDCs of DF and DHF patients on day 3 of illness and compared to that of healthy controls (HC). Data are presented as the median and the statistical analysis of the expression of CD80 and CD86 was performed by the Mann Withney test.*$p< 0.05$, ** $p< 0.01$ and ***$p< 0.001$

Type I IFN production through TLR9 is impaired in DENV infection of PBMCs in vitro

It is known that DENV is a weak inducer of type I IFN production after infection of human DCs. Several possible mechanisms have already been proposed to explain this observation (Mazzon, Jones, Davidson, Chain, & Jacobs, 2009; Munoz-Jordan, Sanchez-Burgos, Laurent-Rolle, & Garcia-Sastre, 2003). The differential expression profiles of TLR3 and TLR9 in DF and DHF patients presented here may also influence type I IFN secretion. However, whether recognition of viral components by TLRs triggers IFN production in DENV-infected cells or, whether DENV infection affects the function of TLRs is largely unclear. To expand our knowledge on this subject, we tested the ability of PBMCs to respond to the TLR3, 4, 7/8 and 9 ligands, and trigger the IFN-α pathways, after challenge with wild-type DENV or iDENV at a MOI of 5 2 hours after the infection. We decided to use PBMCs for these studies to mimic natural infection thereby allowing cross talking between cell subsets. PBMCs only treated with the TLR3, 7 and 9 agonists and challenged with DENV showed a variable but enhanced IFN-alpha production when compared to that of non-treated cells, except for the TLR4 agonist LPS, which produced similar levels of IFN-α as the control (Fig. 7). PBMCs infected with DENV in presence of the TLR3, and 7/8 agonists further stimulated IFN production ($P<0.05$). The observation that iDENV produces a more robust IFN-α response than wild-type DENV is in line with prior published data indicating that the, NS2B, NS4B and NS5 proteins are responsible for inhibition of type 1 IFN production [37-39]. Interestingly, iDENV infection of PBMCs in the presence of the TLR9 agonist CpG

led to a significant decrease of IFN-α expression (P<0.05), compared to PBMCs treated with CpG A only. In addition, PBMCs were treated with TTAGGG ODN, a compound that can neutralize the stimulatory effect of CpG A and consequently the production of IFN-α. After the treatment INF-α production was evaluated and a strong decrease (p<0.05) in IFN-α realease was observed compared with the mock without antagonist (Fig. 7). Were the cells were treated with CpG A, the antogonist and DENV, the IFN-α production was also decreased, suggesting a possible synergistic effect between DENV and the antagonist, in the IFN-α reduction.

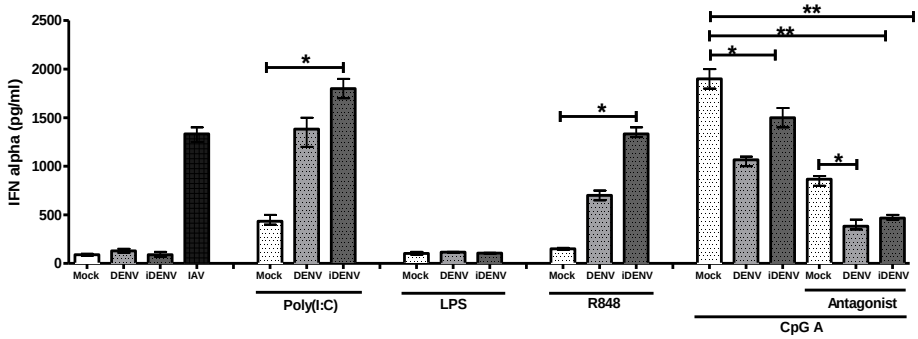

Figure 7. IFN-α production by PBMCs in response to TLR9 ligand is impaired by DENV infection. PBMCs from healthy controls were challenged with wild type DENV or iDENV at a MOI of 5 and then stimulated with agonists for TLR3 (poly:IC), TLR4 (LPS), TLR7 and TLR8 (R848) and TLR9 (CpG A). The production of IFN-α was measured by ELISA after 24 h pot stimuli. The antagonist of TLR9 (TTAGGG ODN) was used to neutralize the stimulatory effect of GpG A in the presence of DENV or iDENV. The supernatant from C6/36 cells were used as mock and Influenza virus was used as positive control for IFN-α production.Data are presented as the mean of values of at least two independent experiments performed in triplicate; error bars indicate standard deviation. Statistical comparisons among groups were carried out using the Kruskal-Wallis test comparing between groups. *p<0.05 and** p<0.01.

Discussion

An immune response with loss in the homeostasis of cytokine secretion has been proposed as the central event in the development of DHF (Pang, Cardosa, & Guzman, 2007). TLRs are important initiators of cytokine production and in this report we show that DENV can modulate/alter TLRs expression in DCs. We observed an increased expression level of TLR3 and TLR9 in mDCs and of TLR2 in pDCs during the acute phase (days 3-5 after onset of symptoms) of DENV infection. When dengue patients were classified according the clinical outcome, a higher expression level of TLR2 was seen in DHF patients when compared to DF patients. In contrast, mDCs of DF patients expressed higher levels of TLR3 and TLR9 than those of DHF patients especially at day 5 of illness. In pDCs, this difference was also observed for TLR9 on day 3 of illness.

The mDCs and pDCS are both important players of the innate immune system but vary with respect to their origin, phenotype and functional features (Dudziak et al., 2007; Steinman & Cohn, 1974). Previous studies have also indicated that these cells differ in permissiveness to DENV infection. Whereas mDCs are readily infected with DENV, pDCs are essentially non-permissive to infection (Sun et al., 2011).Interestingly, we observed changes in TLR expression in both mDCs and pDCs during the early acute phase of illness. TLR3 expression was increased in mDCs and this is likely related to infection of these cells since TLR3 is known to recognize dsRNA (Sun et al., 2011). Cell surface-expressed TLR2 was up-regulated in pDCs, presumably due to sensing of the virus at the cell surface. Indeed, even though pDCs do not support a full replicative cycle of DENV, these cells do respond to infection since phenotypic and functional changes have been described upon addition of DENV to these cells (Steinman & Cohn, 1974). This phenomenon has also been previously reported for HIV-1, where in vitro studies showed up-regulation of TLR2 and TLR4 in pDCs (Hernandez, Arteaga, Paul, Kumar, Latz, & Urcuqui-Inchima, 2011b; Hernandez, Stevenson, Latz, & Urcuqui-Inchima, 2012).

Notably, we also found an up-regulation of TLR9 in mDCs. Basal expression of TLR9 has not been previously reported on mDCs, but a recent study showed that mDCs respond to the TLR9 agonists CpG A and CpG B thereby increasing the expression levels of CD80 and of human leukocyte antigen (HLA-DR9) molecules on the cell (Nguyen et al., 2010). These findings suggest that the expression of TLR9 can be enhanced after stimulation with its agonist. We show here that IFN-α production was significantly affected in PBMCs exposed to DENV in the

presence of the TLR9-agonist, suggesting that TLR9 responds to DENV infection.However, how TLR9 is activated in DENV infection is unclear,as TLR9 is known to recognize pathogen-associated DNA (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Hemmi et al., 2002; Krieg, 2002). One could speculate that the increased TLR9 expression is due One could speculate that the increased TLR9 expression is due cross-talking between different receptors or adaptor proteins from different signal pathways as this has been described to occur in a pro-inflammatory environment (Yoshida, Jono, Kai, & Li, 2005).

Monocytes have also been reported to represent target cells of DENV replication (Sun et al., 2011). Moreover, Azeredo et al. (Azeredo et al., 2010) showed an increase of TLR2 expression in monocytes CD14+ of dengue-infected patients. Subsequent in vitro studies revealed an increased frequency of TLR2 in pro-inflammatory monocytes (CD14+ and CD16+) and it was proposed that overexpression of TLR2 in CD16+ cells could contribute to DHF. We also assessed TLR2/TLR4 expression in monocytes but no effect of DENV infection on TLR2 and TLR4 expression was detected. This discrepancy is probably related to differences in the analysis and classification of the cell populations.

TLR3 is specifically up-regulated in mDCs in patients with acute DF since no TLR3 up-regulation is seen in patients that develop DHF. Previously, Tsai and co-workers (Tsai et al., 2009) reported that TLR3 induces an anti-dengue response in HEK293 cells. Furthermore, there is evidence that the type I IFN response initiated by TLR3 contributes to the elimination of Hepatitis C virus (Kanda, Steele, Ray, & Ray, 2007). Based on the above observations, we postulate that TLR3 is associated with antiviral responses towards DENV. by TLR3 could promote the production of pro-inflammatory cytokines and IFN. This inhibiting DENV replication and therefore could prevent the development of severe manifestations. Indeed, and in line with our results, a recent report showed down-regulation of TLR3/4/7 in PMBCs of patients experiencing secondary DHF but not DF (Modhiran et al., 2010).

TLR2 expression was up-regulated in pDCs and mDCs of DHF patients but not of DF patients. Recent studies suggest that TLR2 is an important promoter of pro-inflammatory cytokine release, reviewed in (Mangada & Rothman, 2005; Marshall, Heeke, Gesner, Livingston, & Van Nest, 2007; Yoshida et al., 2005). Increased pro-inflammatory cytokine production is one of the hallmarks of severe disease (Mangada & Rothman, 2005; Suharti et al., 2003). Therefore we postulate that individuals with a higher response to DENV through TLR2 may be more likely to develop severe manifestations, whereas patients who express TLR3 and TLR9, may have some degree of protection and may probably be less likely to develop DHF.

TLRs not only promote cytokine release; they also promote the up-regulation of co-stimulatory molecules such as CD80 and CD86 to favor cell maturation and efficient antigen presentation to T cells (Akira & Takeda, 2004). We found that mDCs of DHF patients have lower expression levels of CD80 and CD86 than HC suggesting inefficient maturation of mDCs in these patients. This may be explained by the low TLR3 and TLR9 expression levelin patients with DF or DHF. In pDCs, a significant down-regulation of CD86 was observed in both DF and DHF patients. This could have important consequences in the development of a specific immune response able to induce memory T cells to control future infection. This hypothesis is supported by the observation of lower CD80/CD86 expression levels in DHF patients, compared to DF patients (in mDCs) and healthy donors (in DCs). Libraty et al. (Libraty et al., 2001) reported lower expression of CD80 and CD86 in DCs in DENV infection in vitro. Sun et al. (2009) observed that in purified pDCs and mDCs the presence of the virus promotes the expression of CD80 and CD86 in mDCs but not in pDCs suggesting that DENV can down regulate the co-stimulatory molecules CD80 and CD86 (Sun et al., 2011). However, further studies are required to elucidate the mechanisms involved in this phenomenon.

In conclusion the differential expression of TLRs in dengue may influence the clinical outcome of the disease. Future research is necessary to fully understand the participation of the innate immune response in dengue pathogenesis and to assess the possibility of using TLR agonists as vaccine adjuvants in dengue vaccines.

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Chapter 5

Altered immune response of immature dendritic cells upon dengue virus infection in the absence and presence of antibodies

Silvia Torres, Heidi van der Ende- Metselaar, Silvio Urcuqui-Inchima, Jolanda M. Smit, and Izabela Rodenhuis-Zybert

Dendritic cells (DCs) play a dual role in DENV infection. They are the major initiators of antiviral immune responses and also susceptible to be infected. Previous observations named DCs as major targets in DENV infection, and show that infected cells can undergo maturation and cytokine productions under infection conditions. We evaluate the maturation profiles of DENV in immDCs infected with DENV in the presence or absence of DENV-immune serum. We found that DENV infection hampers the ability of DCs to unregulated cell surface expression of the co-stimulatory markers CD83 and CD86, and the major histocompatibility complex molecule HLA-DR when assessed by flow cytometry. Low concentrations of IL4, IL6, IL10 and TNF-α were found in supernatants from cells infected unaided to antibodies when compared to the cells infected in neutralizing conditions, Suggesting that the presence of immuno-complexes activates signalization and cytokine production.These results demonstrate that DENV impairs response by DCs an suggest that the extra stimuli by high concentrations of antibodies can works as compensatory mechanism to restore the DCs response, highlighting the protective role of antibodies during DENV infection.

To be submitted in Plos Negl Trop Dis

Author summary:

DCs have been proposed as major targets in DENV infection and are, without doubt, a critical component of the initiation of antimicrobial response. Here we evaluated the susceptibility and response of DCs in the presence and, in the absence, of DENV-immune serum. We found that the internalization of viral particles detected by flow cytometry was not always indicative of the production of infective particles. Thereafter, when we evaluated the effect of antibodies in the phenotype and function of DCs we found that DENV infection, unaided to antibodies, blocked the maturation and cytokine production of immDCs. In contrast, the presence of DENV-antibodies complexes induced maturation of the cells, as well as cytokine production. We concluded that direct infection by DENV blunted the innate immune response. The upregulation of maturation and cytokine productions, carried out in the presence of immune-complexes formed during neutralizing conditions, could play a crucial part in the host's defense against dengue virus, favouring the neutralization of the infection and, consequently, avoiding disease progression.

Introduction

With an estimated 400 million infections each year, dengue virus (DENV) is the most important mosquito-borne viral disease world-wide. There are 5 serotypes of DENV (DENV1—5) and infection with each serotype can be asymptomatic or lead to a wide range of clinical symptoms ranging from a flu-like illness to severe and potentially fatal disease (Bhatt *et al.*, 2013; ScienceInsider, 2013; WHO, 1997). Primary infections are often asymptomatic and provide life-long protection against homotypic re-infection and short term (approx. 9 months) protection against heterotypic re-infections (revised in (Murphy & Whitehead, 2011)). Intriguingly, individuals that are re-infected with a heterologous virus serotype after the cross-protection period are at risk of developing severe disease (Halstead, 2003; Halstead, 2007). Severe disease is also seen during primary infections in infants with low numbers of circulating maternal dengue antibodies (Simmons *et al.*, 2007). Thus, antibodies appear to play an important role in controlling DENV infection. Indeed, *in vitro* and *in vivo* studies demonstrated that DENV infectivity depends on the concentrations and/or avidity of antibodies present during infection. DENV infection is contained in the presence of neutralizing antibody titers (de Alwis *et al.*, 2011; Endy *et al.*, 2004; van der Schaar *et al.*, 2009). At sub- and non-neutralizing antibody concentrations, however, enhanced infection of immune cells is seen by facilitating viral entry via FcR-antibody interaction (Halstead, 2003).

Dendritic cells (DCs) are the front line of defense invading pathogens. Upon antigen recognition immature DCs (immDCs) acquire a mature phenotype (matDCs), which is characterized by high cell surface expression of co-stimulatory molecules, major histocompatibility complex II molecules (HLA-DR), as well as secretion of pro- and anti-inflammatory cytokines (Banchereau & Steinman, 1998; Cella *et al.*, 1997). Activation of DCs is crucial for shaping the innate responses as well as for initiation of adaptive immunity (Banchereau & Steinman, 1998; Cella *et al.*, 1997). DCs also represent the primary targets for DENV replication during primary and secondary infections (Marovich 2001; Wu *et al.*, 2000). In the absence of antibodies (direct infection), immDCs have been shown to be particularly permissive to DENV infection (Boonnak *et al.*, 2008; Boonnak *et al.*, 2011; Nightingale *et al.*, 2008) and viral replication was shown to blunt their maturation cytokine production (Chang *et al.*, 2012; Munoz-Jordan *et al.*, 2003; Munoz-Jordan, 2010). In the presence of high antibody titers, immDCs were shown to neutralize viral infection (Boonnak *et al.*, 2008). ImmDCs do not support enhancement of infection at decreasing antibody concentrations (Boonnak *et al.*, 2008). Notably, the effect of DENV-specific antibodies on the activation of DCs upon infection remains unknown.

In the present study, we evaluated the infectious properties of DENV in immDCs in the absence and presence of dengue-specific antibodies and investigated the effect antibodies on immDCs activation. We show that immDCs are only moderately permissive to DENV infection as the majority of the internalized virions do not induce a productive infection. We show that in the absence of antibodies DENV replication inhibits the ability of immDCs to undergo maturation and produce cytokines. On the contrary, depending on the antibody titers, DENV-immune complexes triggered fully or partially mature DCs phenotypes with distinct cytokine profiles, suggesting yet another mechanism by which pre-existing antibodies may influence DENV infection.

Methods

Virus and cell lines

DENV-2 strain 16681 was propagated in the Aedes albopictus cell line C6/36 as described before (Rodenhuis-Zybert *et al.,* 2010). In summary, C6/36 cells were inoculated with DENV-2 at a multiplicity of infection (MOI) of 1. DENV particles were harvested at 72 hours post-infection (hpi) and cleared from cell debris by low speed centrifugation. The specific infectivity of DENV-2 was determined by measuring the number of infectious units by plaque assay on BHK-15 cells and the number of genome containing particles (GCPs) by quantitative PCR (qPCR) analysis, as described previously (van der Schaar *et al.*, 2007; Zybert *et al.,* 2008). To generate UV-inactivated (UVi) virus, 1mL of DENV-2 was placed in a petri dish and exposed to UV light for 1 hour. The reduction in specific infectivity of UVi-treated virus was confirmed by plaque assay and qPCR, as described before (van der Schaar *et al.,* 2007; Zybert *et al.*, 2008).

Antibodies and DENV-immune serum

Intracellular DENV staining was done by using the murine anti-DENV 3H5 (Millipore) or the rabbit anti-DENV orb1707 (Biorbyt). A well-characterized convalescent DENV-2 immune serum (kindly provided by G. Comach Biomed-UC, Lardidev, Maracay, Venezuela; and T. Kochel, U.S. Naval Medical Research Center Detachment, Lima, Peru) was used as a source of polyclonal antibodies. The serum was obtained from a 12 year old boy with a primary DENV2 infection and was collected at 28 days after the onset of disease symptoms.

Monocyte-derived dendritic cells

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy coats by density centrifugation using Ficoll-Paque™ Plus (GE Healthcare). The buffy coats were obtained with informed consent from healthy volunteers, in line with the declaration of Helsinki (Sanquin Blood bank). PBMCs were adhered to tissue culture dishes and after 90 minutes, monocytes were isolated by gelatin adherence, as described before (Miller *et al.*, 2008). Next, monocytes were allowed to differentiate in complete RPMI medium (CM) with 20% fetal bovine serum (FBS), 500 U/ml recombinant granulocyte macrophage colony-stimulating factor (rGM-CSF), and 250U/ml recombinant human interleukin-4 (rIL-4) (both from Prospec-Tany). The CM was replaced every second day for 6 days to generate immDCs. After 6 days of culture at 37°C, the phenotype of the cells was confirmed using flow cytometry analysis, as described in Boonnak K (Boonnak *et al.*, 2008). A typical immDCs phenotype corresponds to Lin 1^{neg}, HLA-DR^{pos}, and $CD11c^{pos}$. To generate mature dendritic cells (matDCs), the immDCs were further stimulated with monocyte-conditioned medium mimic (MCMm) containing recombinant human interleukin 6 (rIL-6), recombinant human interleukin 1β (IL-1β), tumor necrosis factor alpha (TNF-α), and prostaglandin E_2 on day 6, as described in (Boonnak *et al.,* 2008). Maturation of DCs was verified on the basis of an increase of the intensity of expression of CD40, CD83, and CD86 surface markers. Representative dot plots of the phenotype of immDCs and matDCs can be found in supplementary figure 1.

Infection assays

DENV-IC were pre-formed by incubating the virus for 1 hour at 37°C with 10-fold sequential dilutions of DENV-2-immune serum. Then, the DENV-immune complexes were diluted with cell culture medium containing 2% FBS and added to immDCs. DENV infection was performed at a multiplicity of infection (MOI) of 0.1, 1, and 10 in the presence or the absence of the diluted DENV immune serum. At 1 ½ hpi at 37 ^{0}C, the inoculum was removed, cells were washed, and medium was added. At 24 hpi, cells and supernatants were harvested and separated by low-speed centrifugation. Cell viability was evaluated using LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen). The phenotype of the cells and viral titers were analyzed using flow cytometry as mentioned below. Virus production was assessed by plaque assay on BHK-15 cells and qPCR analysis (van der Schaar *et al.,* 2007; Zybert *et al.,* 2008).

Flow cytometry

Staining of infected and mock-infected immDCs was performed 24 hpi. In parallel, typical DCs surface markers (including maturation markers) were analyzed. To this end, aliquots of (5×10^5) cells were placed into cytometry tubes with FcRs block buffer True Stain FcX (Biolegend) and incubated for 10 min at room temperature. Cells were subsequently washed in a staining medium containing EDTA, saponin, and 2% FBS, all of which were provided by Sigma. Cells staining was performed with a panel of DCs markers (Lineage cocktail Lin-1 FITC, CD11c A PC, and HLA-DR V450) and a maturation panel (CD 40 alexa fluor, CD83 PECy7and CD86 V500) for 30 minutes at 4°C. Cells were washed twice with staining medium and fixed prior to acquisition. Additionally, a second aliquot was used for intracellular DENV staining. The cells were treated with intracellular fixation buffer in conjunction with the permeabilization buffer (eBioscience). Then, murine anti-DENV 3H5 (Millipore) was added followed by a secondary anti-mouse Alexa-647 labeled antibody. As an additional strategy for viral detection, a rabbit anti-DENV orb 1707 (Biorbit) was used. Isotype controls mAbs were used in every experiment to establish background staining and to set quadrants before analysis. The multi-color FACS analysis was performed on a LSR-II instrument (BD Biosciences) and the analysis was performed using Flow-jo software (Tristar).

Measurement of cytokine levels

Cytokines were measured in cell-free supernatants by using the cytometry bead array Human Cytokine Kit (BD Biosciences) per the manufacturer's instructions. Briefly, multiscreen 1.2µm hydrophilic filter plates (Millipore) were pre-wet with wash buffer and aspirated. Capture beads for each of the four examined cytokines (IL-4, IL-6, IL-10, TNF-α) were combined with 50 µl of supernatant obtained from the infectivity experiments. After 1 hour incubation at room temperature, phycoerythrin detection reagent for each cytokine was pooled and added to the wells. Incubation was continued for 2 hours at room temperature, after which the plate was washed and the beads were re-suspended in wash buffer. Data collection was done on a BD FACS canto cytometer. Calculations were performed using BD Biosciences CBA software.

Statistical analyses

Mann-Whitney U test was applied to the data with Prism software (GraphPad Software Inc). Differences were considered statistically significant when $p<0.05$(*), $p< 0.01$ (**), $p<0.001$(***).

Results

Permissiveness of immDCs to DENV infection in the absence and presence of antibodies

ImmDCs were generated from human monocytes, as described in the methods section. The obtained cell culture was characterized as Lin-1^{neg}, HLA-DR^{pos} and, $CD11c^{pos}$, confirming the differentiation of the cells from monocytes into DCs (Supplementary Figure 1A). To evaluate the ability of the cells to undergo maturation, immDCs were stimulated with monocyte-conditioned medium mimic (MCMm) and stained for surface expression of maturation markers (CD40, CD83, CD86, HLA-DR). At 24 hours post-stimulation, DCs showed increased expression of these maturation markers when compared to non-stimulated cells, confirming the capacity of the immDCs to mature (Supplementary Figure 1B).

A B

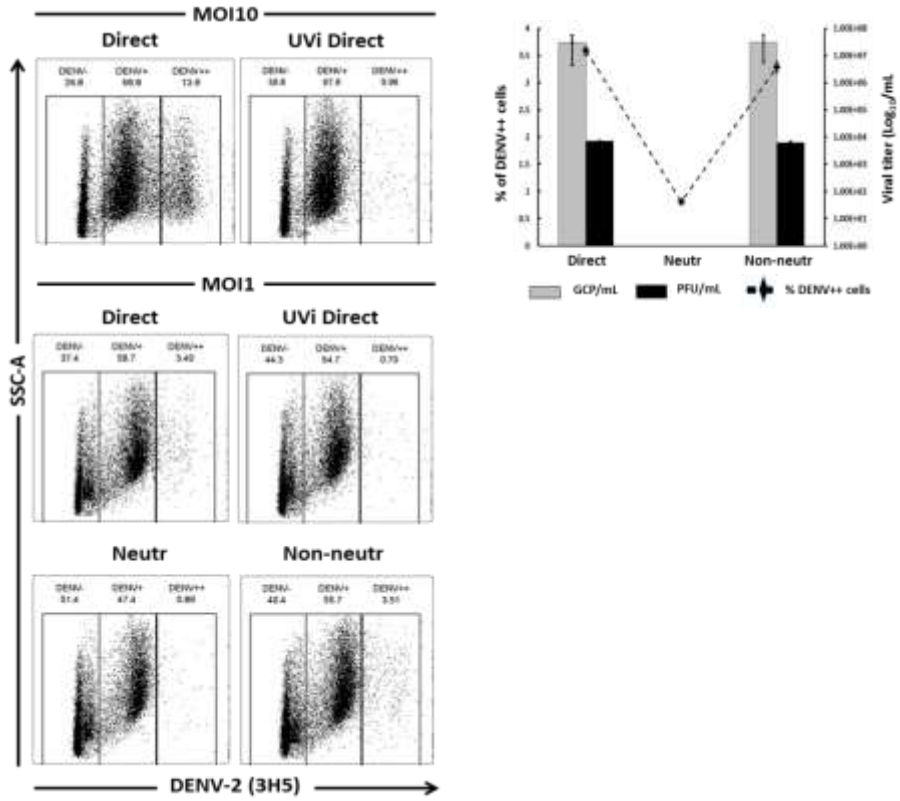


Figure 1. DENV infection of immDCs in the absence and presence of DENV-immune serum. (A) ImmDCs were infected with standard (std) or UV inactivated (UVi) DENV at MOI 1 or MOI 10. Direct denotes DENV infection in the absence of DENV-immune serum. Neutr denotes DENV infection in the presence of serum at dilution 10^3. Non-neutr denotes DENV infection at serum dilution 10^8. Cells and cell supernatants were harvested at 24 hours post-infection (hpi). The frequency of DENV-positive cells was determined using MAb 3H5. DENV-positive populations are designated as DENV+ and DENV++. Representative dot plots of three independent experiments are shown. (B) Quantitative analysis of the infectious properties of DENV in immDCs. Dashed line: percentage of DENV ++ cells; grey bars: number of infectious particles (Log_{10} PFU/ml); black bars: number of genome-containing particles (log_{10} GCPs/mL). Results are representative of at least 10 experiments with 3 donors.

First, we assessed the permissiveness of immDCs to DENV infection (Figure 1). The cells were infected with DENV at MOI 10, 1, 0.1 with or without prior opsonization with increasing dilutions of DENV-2 immune serum. At 24 hpi, the percentage of dengue antigen-positive cells was evaluated by using two distinct DENV-specific mAbs, 3H5 and orb1707. In addition, virus progeny production was measured by plaque assay on BHK-15 cells and qPCR to obtain both the number of infectious (plaque forming units-PFU) and physical particles (genome containing particles -GCPs) titers, respectively. A representative dot plot of immDCs infected at MOI 10 and MOI 1 and stained with DENV-E-specific 3H5 mAb is shown in Figure 1A. Note that two distinct DENV-positive populations (designated DENV+ and DENV++) were

detected following infection at MOI 10 and 1. We detected on average 3 % DENV++ immDCs following infection at MOI 1, which is considerable less than described in literature (Figure1 A and 1B, (Boonnak *et al.*, 2008; Boonnak *et al*., 2011; Marovich *et al* 2001; Nightingale *et al*., 2008)). We did not detect DENV++ cells following infection at MOI 0.1 (data not shown).Since pathogen recognition and antigen presentation is an important feature of DCs, we inferred that the DENV+ population corresponded to cells that internalized the virus but aborted infection, while DENV++ population represented cells that were productively infected. To validate this, we exposed the cells to UVi-DENV at MOI 10 and 1 (inactivation of the virus was confirmed by PFU and GCP titration; data not shown). As shown in Figure 1A and 1B, we could only detect DENV+ cells following exposure of the cells to UVi-DENV (UVi direct panels). This indicates that the population identified as DENV+ populations indeed corresponds to cells that have internalized the virus, whereas the DENV++ populations indicate cells that are actively infected with DENV.

In order to test the effect of DENV-immune serum on the permissiveness of immDCs to DENV, the cells were infected with DENV-2 pre-opsonized with increasing dilutions of the serum at MOI 1. The DENV-immune serum used exhibited potent neutralizing activity and inhibited DENV infection at a serum dilution of 10^2 to 10^6 (data for 10^3 dilution is shown in Figure 1A and 1B). We used immDCs derived from 3 different donors and in none of them we detected PFU or GCP titers (detection limits are 20 PFU/ml and 400 GCP/ml) following infection with DENV pre-opsonized with a 10^3 serum dilution. Therefore, we used this dilution for further neutralization experiments. Opsonization of DENV-2 with serum dilutions 10^7 to 10^{11} led to a recovery of DENV PFU titers to the levels similar as direct infection. In line with a previous report (Boonnak *et al.*, 2008), no enhancement of infection was observed, even at further dilutions of the serum (data not shown). Importantly, as in case of UVi virus, we did detect DENV+ cells under neutralizing conditions (Figure 1A). This finding further corroborates that the DENV+ population represents DCs that internalize the virus but abrogate viral replication. The identification of DENV+/++ populations upon infection of immDCs has not yet been described and we wondered whether this was related to the staining antibody used. Therefore, we also evaluated DENV infection by using a rabbit anti-DENV orb1707. With this antibody, the DENV+ and DENV++ populations were not clearly separated (Supplementary Fig. S2), however, the total percentage of DENV positive cells detected by rabbit anti-DENV orb1707was similar to the sum of DENV+ and DENV++ populations detected by the murin anti-DENV 3H5 Ab (Table 1). Thus, the percentage of DENV positive cells detected by the rabbit anti-DENV orb1707 did not represent active infection but cells that internalized and/or replicated the virus, and implies that immDCs are not so permissive to dengue infection as previously considered.

Changes in DCs phenotype following DENV infection with and without antibodies

The effects of direct DENV infection on the maturation and activation of immDCs has been studied extensively (Boonnak *et al.*, 2008; Boonnak *et al*., 2011; Ho *et al.,* 2001; Libraty *et al., 2*001; Nightingale *et al.*, 2008; Sun *et al.*, 2009). In the majority of these studies, activation of DCs was measured at 48 hpi, which translates to 2 rounds of DENV replication. However, to directly assess the effect of antibodies on DCs maturation it is important to evaluate the expression markers after

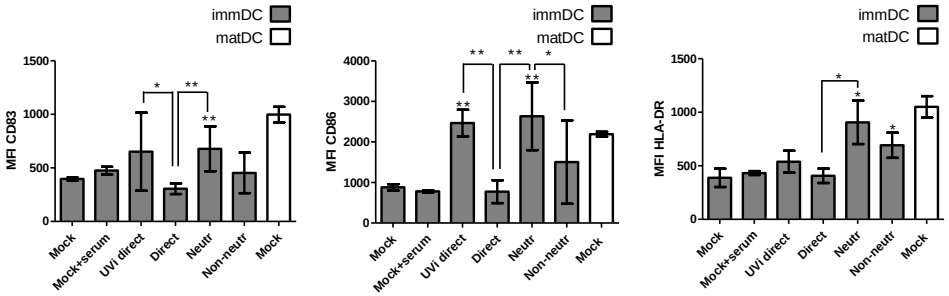


Figure 2. Differences in DC phenotype following direct DENV infection and infection in the presence of antibodies. ImmDCs were infected with std virus or UVi DENV at MOI 1 in the absence and presence of DENV-immune serum. The mean fluorescence intensity (MFI) of the expression of co-stimulatory markers CD83 and CD86 and the major histocompatibility complex molecule HLA-DR was assessed by flow cytometry at 24 hpi. As a control mock-infected matDCs were used (white bars). A representative analysis of at least three independent experiments ± SD is shown. Statistical analysis was done by use of Mann-Whitney U-test (*P< 0.05 ** P < 0.01). Stars above the bars indicate differences when compared to mock-infected cells.

1 round of replication. Therefore, we here measured the surface expression of CD83, CD86, HLA-DR and CD40 at 24 hpi. Mock-infected (with and without the addition of dengue-immune serum) and MCMm-stimulated matDCs were added as negative and positive controls, respectively. CD40 was found to be equally up-regulated in all infection conditions (data not shown). CD83, CD86, or HLA-DR were not up-regulated following direct DENV infection when compared to mock-infected cells (Figure 2). A significant increase in the surface expression of CD83, CD86 and HLA-DR molecules was however observed following exposure of the cells to DENV pre-opsonized with neutralizing serum titers. At neutralizing conditions, the expression levels of the maturation markers were similar to that of MCMm-stimulated matDCs. To investigate, whether the up-regulation of the maturation markers was solely due to the blockage of replication, we compared the expression of the maturation markers in cells infected with DENV at neutralizing conditions with that of cells exposed to UVi-DENV (UVi direct). As depicted in Figure 2 and Supplementary Table S1, there were no clear differences in the expression levels of CD83, CD86 between these two conditions. Expression of HLA-DR was only up-regulated following infection in the presence of neutralizing antibody titers, suggesting that HLA-DR up-regulation is triggered by the DENV-immune complex. To verify this, we next compared the expression profile of maturation markers upon direct infection and infection at non-neutralizing conditions. At these conditions, a similar number of infected cells is seen (Fig.2). Notably, only DENV in complex with antibodies triggered significant up-regulation of HLA-DR, CD83 and CD86 in DCs. Accordingly, it appears that antibody opsonization of DENV provides an extra signal that is essential for immDCs to acquire a fully mature phenotype under neutralizing and an incomplete/semi-mature phenotype under non-neutralizing conditions.

Expression of maturation markers increased only in DCs that internalized the virus.

Previous studies reported differences in the maturation profile of DENV positive and bystander cells (Palmer *et al.,* 2005). Therefore, we sought to evaluate the expression levels of surface molecules in cells that internalized (DENV+), replicated the virus (DENV++) and DENV negative cells (DENV neg). However, all the antibodies available to determine the maturation profile of DCs are generated in mice, and therefore the detection pair comprising murine anti-DENV 3H5 clone and anti-mouse Ab could not be used. As primary labeling of this Ab proved not efficient enough to visualize the DENV+ and DENV++ cell populations, we used the rabbit anti-DENVE orb1707 Ab (Table 1). As demonstrated in Figure 3, in all infection conditions DENV antigen-positive DCs expressed higher levels of maturation markers when compared to negative as well as mock-infected cell populations. Nonetheless, the DENV

antigen-positive cells (black bars) detected following exposure to abortive infection (neutr. and UVi conditions) expressed

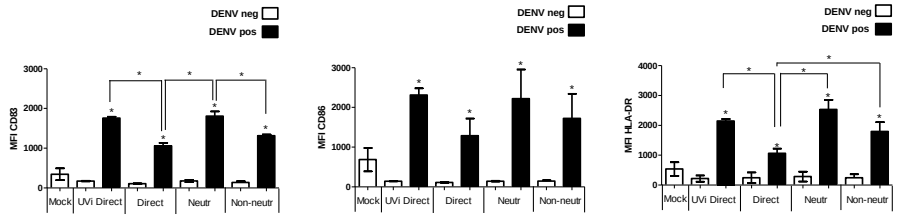


Figure 3. DENV antigen-positive and negative cells differentially express maturation markers. Experimental conditions and subsequent analysis are similar as described in the legend to Figure 2. DENV infection was done at MOI 1. Cells were stained with a rabbit anti-E antibody. White bars: DENV antigen-negative cells; black bars: DENV antigen-positive cells. The data is representative of three independent experiments ± SD. Statistical analysis was done by use of Mann-Whitney U-test (*P< 0.05). Stars above the bars indicate differences when compared between DENV positive and negative cells in each condition tested

relatively higher levels of maturation markers than those exposed to successful infection(direct and non-neutr. conditions). This suggests that cells that actively replicate DENV express lower levels of maturation markers when compared to cells that only internalized virus. In line with the data from the total DCs population, significantly higher expression of HLA-DR was found in DENV antigen-positive cells exposed to DENV in complex with antibodies, than in those exposed to direct infection (Figure 3).This strengthens the idea of a distinct DCs activation pathway in the presence of immune-complexes.

Presence of specific antibodies during DENV infection alters the profile of cytokines released from DCs.

Activated DCs release soluble immune modulators including cytokines, which play an essential role in shaping the immune response (Banchereau & Steinman, 1998). Accordingly, we next investigated whether differences in the level of DC maturation induced after DENV infection in the absence and the presence of antibodies translated to a particular cytokine expression profile. Herein, we evaluated the levels of pro- (IL-6, TNF-α), and anti-inflammatory cytokines (IL-10, IL4), in the supernatants of DCs infected at different conditions (Figure 4). Upon direct infection, no change in maturation markers was observed and in line with this, no increased levels of cytokine production were seen when compared to the mock-infected cells. Furthermore, immDCs exposed to DENV under neutralizing conditions did mature and secreted significantly higher amounts of TNF-α, IL-4, IL-10 and IL-6. It has been reported that ligation of FcγRIIa is required for production of IL-6 and TNF-α following infection of matDCs under ADE conditions (Boonnak *et al*., 2008). Therefore, we blocked FcγRIIa in immDCs and infected the cells with DENV pre-opsonized with a neutralizing serum dilution. Indeed, blocking FcYRIIa inhibited the production of cytokines as similar levels were observed during mock infection (Figure 4), indicating that FcγIIa ligation triggered the cytokine production. Interestingly however, blocking of FcγRIIa had no effect on the neutralizing capacity of the antibodies (data not shown), suggesting that neutralization and activation depend on the engagement of different Fc receptors and that this occurs simultaneously. At non-neutralizing infection conditions, higher levels of IL-6, IL-4 and TNF-α were found when compared to direct infection. As the addition of the serum alone did not trigger cytokine response (data not shown), these results suggest that opsonization of DENV with non-neutralizing antibody titers triggers this distinct DCs phenotyp

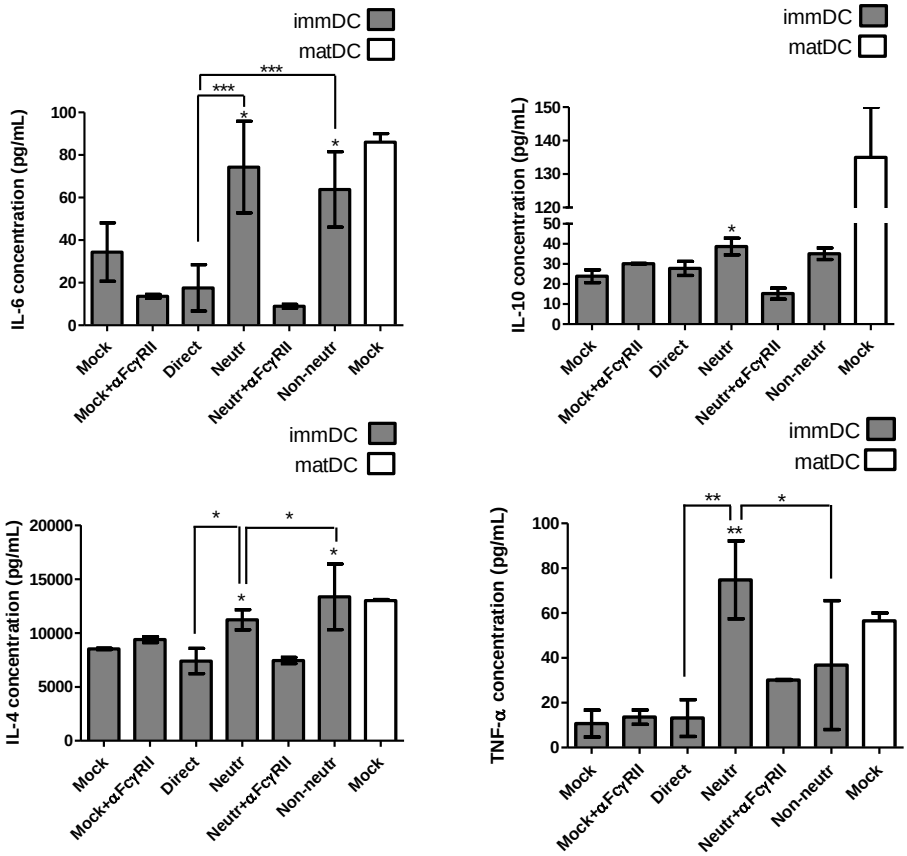


Figure 4. **DENV-immune complexes stimulate cytokine secretion by DCs.** DENV infection (MOI 1) was performed as described in the legend to Figure 2. When indicated, anti-FcγRIIa was added to immDCs prior infection. At 24 hpi, TNF-α, IL-4 IL-6, and IL-10 production was measured by a Cytokine Bead Assay (CBA). White bars: mock-infected cells; grey bars: DENV-infected cells. The data shown are representative of at least three independent experiments ± SD. Statistical analysis was done by use of Mann-Whitney U-test (* P< 0.05; ** P < 0.01, *** P <0.001). Stars above the bars indicate differences when compared to mock.

Discussion

In the present study, we investigated the susceptibility of immDCs to DENV in the absence and presence of DENV-immune serum and evaluated their response to infection. By a combinatory approach of different titration methods we showed that many immDCs internalize DENV particles but only a relatively small fraction of cells is productively infected. This indicates that the majority of immDCs aborted viral infection. DENV-immune serum, depending on the serum dilution, neutralized DENV infection or had no effect. Yet, we found substantial differences in the maturation and response of immDCs infected with DENV at neutralizing or non-neutralizing antibody conditions. In the absence of antibodies, DCs were not activated. In contrast, however, at conditions of antibody-mediated neutralization of DENV infection, immDCs matured and a balanced inflammatory response was seen. At non-neutralizing antibody conditions, DENV replication proceeded and resulted in partial DCs maturation with a more inflammatory cytokine pattern.

It is generally believed that immDCs are highly susceptible to DENV infection (Boonnak *et al*., 2008; Dejnirattisai *et al.,* 2011; Ho *et al.,* 2001; Marovich *et al*., 2001; Nightingale *et al.,* 2008; Wu *et al.,* 2000). However, the percentage of DENV positive cells described by the different groups is wide ranging, and this is likely due to differences in the genetic background of the DCs donors, the viral strains used, and the time-post infection at which the analysis is performed (Boonnak *et al.,* 2008; Dejnirattisai *et al.,* 2011; Ho *et al.*, 2001; Marovich *et al.*, 2001; Nightingale *et al.,* 2008; Wu et al., 2000). Interestingly, we herein demonstrate that the majority of immDCs that internalized the virus successfully aborted infection. Only a small fraction of immDCs is productively infected. Flow cytometry was used previoulsy to assess DENV infectivity but only a distinction between DENV antigen-positive and DENV antigen-negative cells was made (Boonnak *et al.*, 2008; Dejnirattisai *et al.*, 2011; Ho *et al.*, 2001; Marovich *et al.*, 2001; Nightingale *et al.*, 2008; Wu *et al.*, 2000). Antibodies binding to DENV structural proteins detect a higher (Marovich *et al.*, 2001; Wu *et al*., 2000) or lower (Dejnirattisai *et al*., 2011) percentage of DENV antigen-positive cells than Abs that bind DENV nonstructural proteins. Thus, apparently, the epitope specificity of the anti-dengue antibody used is of a high importance when analyzing the permissiveness of immDCs to infection. Indeed, only one of the DENVE- specific antibodies that we tested was able to distinguish between an abortive and an active infection. Therefore, based on our results, we believe that the general perception of high susceptibility of immDCs may be overestimated.

The main role of DCs is to sense, process and present antigens of invading pathogens to cells of the adaptive immune system (Banchereau & Steinman, 1998). Yet, many viruses such as HIV-1, measles, vaccinia and DENV target DCs for infection (Boonnak *et al.*, 2008; de Witte *et al*., 2006; Dejnirattisai *et al*., 2011; Ho *et al*., 2001; Liu *et al.,* 2001; Nightingale *et al*., 2008; Rinaldo, 2013; Wu *et al*., 2000). Interestingly, despite this dual role of DCs, thus far none of studies claimed the ability of DCs to abort DENV infection. However, close examination of the results from Nightingale and co-workers revealed that exposure of DCs to UVi DENV leads to a shift in the antigen-specific fluorescence intensity of the cells when compared to mock-infection (Nightingale *et al*., 2008). Regrettably, although their data strongly suggests that the authors could distinguish between abortive and productive infection, this observation was not discussed. Further studies should define the mechanisms by which immDCs abort or support viral infection.

Table1.

Comparison of the percentage of DENV-positive cells by flow cytometry using two distinct DENV E specific antibodies.

Murine anti-DENV 3H5				**Rabbit anti-DENV(orb1707)**
Infection condition	**% DENV+**	**% DENV++**	**% DENV +/++ (DENV pos.)**	**% DENV pos.**
Direct	61.0± 9.84	2.5 ±0.90	63.5 ± 9.72	58.2± 19.0
UVi direct	54.0 ±3.0	0.1 ±0.4	54.0 ± 2.64	52.x±24.0
Neutr	44.03 ±7.0	0.8 ±1.0	45.1 ± 6.45	43.0±16.50
Non-neutr	47.73±8.10	3.3 ±0.2	51.5 ±10.0	57.0±12.2

DENV + : cells that internalized the virus; DENV++: cells that are productively infected with DENV.

Given the pivotal role of DCs in promoting adaptive immune responses, it is not surprising that many viral pathogens employ immuno-modulatory effects to impair the ability of the infected DCs to initiate adaptive immunity (reviewed by (Lilley & Ploegh, 2005). Importantly, however, conflicting reports have been published with regard to the effect of DENV on DC maturation (Ho *et al.*, 2001; Libraty *et al.*, 2001; Nightingale *et al.*, 2008).

A few studies (Ho *et al.*, 2001; Libraty *et al.*, 2001) reported that DENV replication is essential for the induction of cytokine production by DCs whereas others show that DC maturation is blunted upon DENV infection (Chang *et al.*, 2012; Munoz-Jordan *et al.*, 2003; Rodriguez-Madoz *et al.*, 2010). In agreement with the latter groups, we found that DENV replication inhibits DCs maturation. Along with the lack of phenotypic DCs activation, we did not observe an increase in the production of pro- (TNF-α, Il-6) or anti-inflammatory (Il-4, Il-10) cytokines. This suggests infection of immDCs with DENV-2 alone does not trigger the immune responses that are required to develop adaptive immunity. Importantly, direct infection did not trigger production of pyrogenic cytokines including TNF-α and IL-6, what might explain why the majority of primary infections are asymptomatic. In addition, we presented that dengue-specific antibodies may exert distinct immuno-modulatory effects during DENV infection in immDCs. At conditions of antibody-mediated virus neutralization, the expression of HLA-DR, CD83 and CD86 was up-regulated to levels found in matDCs stimulated by MCMm cocktail. Indeed, previous studies showed that loading DCs with antigen-IgG immune complexes (ICs) leads to efficient antigen uptake and maturation of DCs (den Dunnen *et al.*, 2012; Regnault *et al.*, 1999; Schuurhuis *et al.*, 2006).Interestingly, at conditions of DENV neutralization, we also observed increased production of pro-inflammatory (IL-6, and TNF-α) and anti-inflammatory (Il-4 and Il-10) cytokines. The simultaneous production of TNF-α and IL-10 following neutralized infection may suppress overstimulation of the inflammatory responses that contribute to the pathogenesis of severe dengue (Costa *et al.*, 2013; Pang *et al.*, 2007; Soundravally *et al.*, 2013).

Ligation of FcγRIIa was required for the activation of cytokine production by immDCs exposed to DENV pre-opsonized with neutralizing antibody titers. This finding was particularly interesting since FcγRIIa blockage did not abrogate virus neutralization. To date, engagement of FcγIIa in matDCs has been associated with induction pro-inflammatory responses following antibody-mediated enhancement but not during neutralization of infection in mature DCs (Boonnak *et al.*, 2008). Interestingly, recently a study by Chawla *et al* showed that FcγRI binds antibody-opsonized DENV more efficiently than FcγRIIa, however FcγRI is only preferentially engaged by neutralizing, but not non-neutralizing antibody concentrations (Chawla *et al.*, 2013). This may explain our observation that blockage of FcγRIIa had no effect on virus internalization and neutralization. Although our study is the first to infer the importance of FcγRIIa in the induction of DCs activation during likely FcγRI-mediated neutralization, further studies will be needed to substantiate this notion.

Remarkably, exposure of immDCs to DENV at non-neutralizing conditions triggered an significant increase of HLA-DR expression but not that of the co-stimulatory molecules. Moreover, TNF-α, IL-6, and IL-4 but not IL-10 were released at this condition. The presence of IL-4 is known to inhibit IL-10 production by DCs (Yao *et al.*, 2005). Therefore, it is possible that the lack of IL-10 production is due to the significantly elevated levels of IL-4 released from DCs exposed to non-neutralizing conditions, when compared neutralizing conditions. Furthermore, the ability of DENV in complex with non-neutralizing immune sera to increase cytokine production suggests that additional viral entry mechanisms (e.g., mediated by FcR) are used with different signaling components and downstream functional effects.

Herein we showed that presence of antibodies during DENV infection of DCs has consequences for the maturation of DCs and activation of the cytokine responses. Our results corroborate earlier findings that DENV-2 can blunt the maturation and activation of exposed DCs (Palmer *et al.*, 2005). Additionally, we

show that in the presence of high concentrations of antibodies DENV infection in DCs is not only neutralized but it also rescues the ability of DCs to mature and induce cytokine production. Also, our data reveals that this dual effect of neutralizing antibodies is governed by different Fcγ receptors. On the other hand, infection in the presence of non-neutralizing antibody titers may induce a phenotype of DCs that while will lack the ability to initiate adaptive immunity, triggering mainly pro-inflammatory responses. Further studies should investigate whether this partially impaired DCs phenotype contributes to the aberrant T responses and exacerbation of inflammation that hallmark the severe disease (Green & Rothman, 2006; Mangada & Rothman, 2005; Rothman, 2011).

Acknowledgments

The authors thank Aalzen de Haan and Colm Rattigan for critically reading the manuscript and their valuable comments.

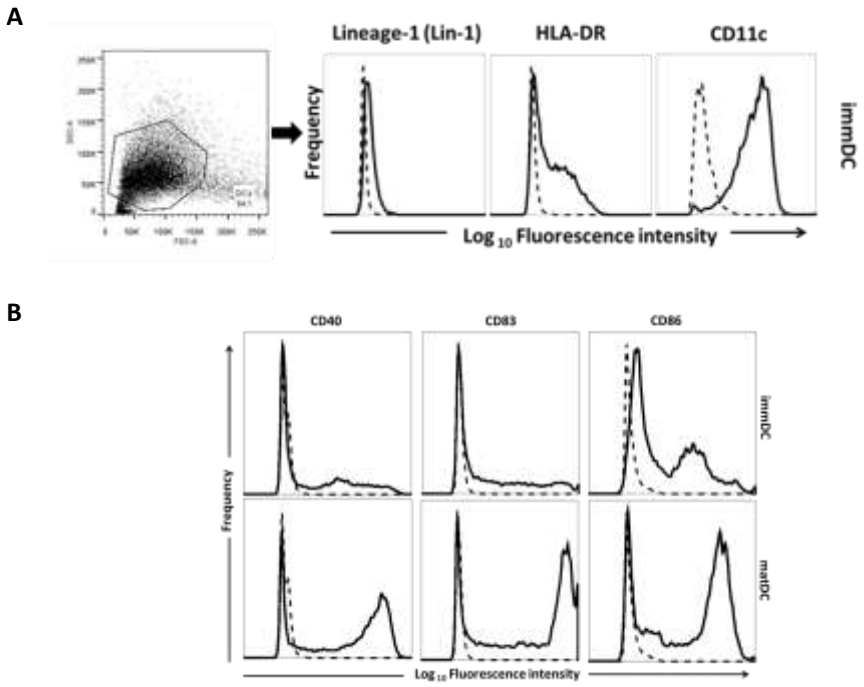
Suplementary tables

Supplementary table 1

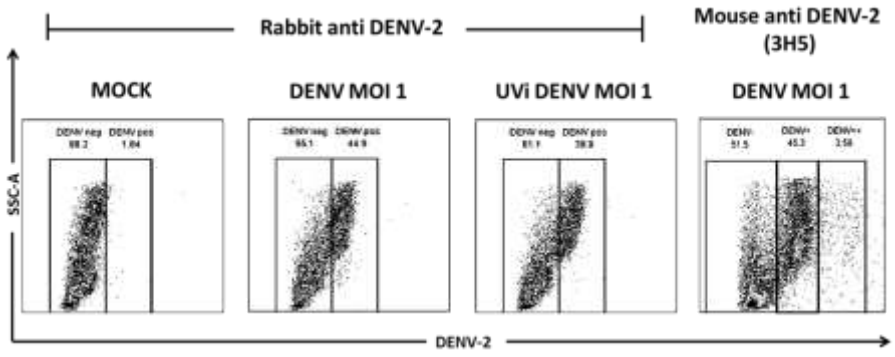
Infection condition	CD83 **(n=3)**	CD86 **(n=3)**	HLA-DR **(n=3)**
Mock	399 ± 12	880 ± 76	533 ± 217
Neutr	678 ± 209[a]	2634 ± 834[a]	905 ± 530 [a]
UVi DENV	652±365	2467±328	538±250

Both infection conditions result in the low number of DENV pos cells and, non-detectable viral production (PFU or GCP/mL)
[a] p<0.5 compared to mock-infected control

Suplementary figures



Supplementary figure 1. Phenotypic analysis of immDC and matDC. (A) Flow cytometry evaluation of monocyte-derived immDC. A total of $1.5x10^5$ cells were gated as immDC. Histograms show the fluorescence intensity of typical dendritic cell markers: Lin-1$^-$, HLA-DR and CD11c. Dashed line: isotype control; normal line: Ab-stained cells. (B) MFI of the co-stimulatory markers CD40, CD83, and CD86 of immDC and matDC.



Suplementary figure 2. Comparison of the percentage of DENV positive cells between the Murine α DENV (3H5) and Rabbit α DENV -2 (orb1707 Biorbit). (A) Immature DC were infected with standard (std), UV inactivated (UVi) DENV at MOI 1. After 24 hours of infection, the frequency of DENV-positive cells was determined using the Murine α DENV (3H5) and Rabbit α DENV -2 (# orb1707 Biorbit). DENV-positive populations are designated as DENV+ and DENV++ for a. Representative dot plots of three independent experiments are shown

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Chapter 6

Dendritic Cells as DENV factories: Immature Vs mature cells

Silvia Torres, Heidi van der Ende- Metselaar Jolanda Smit and Izabela Rodenhuis-Zybert

Dengue virus (DENV) predominantly targets cells of the host immune system for replication. Immature dendritic cells (immDCs), potent sentinels of host immunity, are generally considered to be the most important target cells during DENV infection. ImmDCs have been shown to be highly permissive to DENV infection due to abundant expression of the DENV receptor molecule DC-SIGN at the cell surface. On the contrary, mature DCs (matDCs) express lower numbers of DC-SIGN molecules and hence are believed to be less permissive to direct infection. Interestingly, the decrease in DC-SIGN expression is thought to confer matDCs susceptible to antibody-dependent enhancement (ADE) of DENV infection. ADE is generally believed to underlie the pathogenesis of severe disease following heterologous secondary DENV infections. In this study, we evaluated the infectious potential of DENV particles released from immDCs and matDCs in the absence and presence of antibodies. We show that immDCs, although somewhat more permissive to DENV than matDCs, produce a large number of non-infectious virus particles. Conversely, matDCs support enhanced DENV infection in the presence of non-neutralizing antibodies and produce highly infectious virus. In conclusion, we observed that quantity does not always translate to quality signifying that determination of the specific infectivity of DENV is required to prevent misinterpretation of the importance of different DCs subsets as dengue factories.

Short communication
Manuscript in preparation

Dengue is the most important human viral disease transmitted by mosquitoes worldwide (WHO, 2009). The five DENV serotypes are predicted to cause 390 million human infections each year (Bhatt *et al*., 2013; ScienceInsider, 2013). Approximately 100 million of these individuals present clinical symptoms ranging from an uncomplicated fever to severe hemorrhagic manifestations and/or fatal disease (Bhatt *et al.*, 2013). Mild illness is frequently seen following primary as well as secondary infections (WHO, 1997; WHO, 2009). Heterotypic re-infections and primary infections of infants born to dengue-immune mothers confer a risk for the development of severe disease (Burke & Kliks, 2006; Halstead & Simasthien, 1970; Halstead & O'Rourke, 1977; Simmons *et al*., 2007).These observations underscored the essential role of antibodies in controlling DENV pathogenesis and led to the widely accepted hypothesis of antibody-dependent enhancement (ADE) of infection (Halstead, 2003). According to the ADE theory, antibodies at sub-neutralizing conditions contribute to enhanced DENV infection by directing the virus to Fcγ receptor-expressing immune cells including dendritic cells (Boonnak *et al.*, 2008; Boonnak *et al.*, 2013; Huang *et al.*, 2006; Moi *et al*., 2011). The higher infected cell mass is believed to explain the higher viremia titers found in the blood of patients that eventually develop severe disease.

Dendritic cells (DCs) and macrophages are considered to be main factories of DENV particle production (Marovich *et al.*, 2001; Sun *et al.*, 2009; Wu *et al.*, 2000). When an infected mosquito takes a blood meal, its saliva containing the virus is injected into the skin, where skin-resident immature DCs (immDCs) are believed to be primary targets. At the same time, a substantial number of mosquito-derived DENV particles are delivered directly into the blood, where immDCs, macrophages, and mature DCs (matDCs) are preferentially infected (Blackley *et al.*, 2007; Jessie *et al.*, 2004; Marovich *et al.*, 2001; Wu *et al.*, 2000). DENV has been shown to efficiently infect immDCs due to abundant expression of the DENV receptor molecule DC-SIGN at the cell surface (Boonnak *et al.*, 2011; Tassaneetrithep *et al.*, 2003). Maturation of immDCs leads to down-regulation of DC-SIGN molecules at the cell surface and therefore matDCS are considered less permissive to DENV infection (Boonnak *et al.*, 2008; Boonnak *et al.*, 2011; Wu *et al*., 2000). Interestingly, the decrease in DC-SIGN expression is thought to confer matDCs susceptible to ADE of DENV infection in the presence of non-neutralizing antibodies (Boonnak *et al.*, 2008). Indeed, immDCs do not support ADE unless the expression of DC-SIGN is dowregulated (Boonnak *et al.*, 2008).

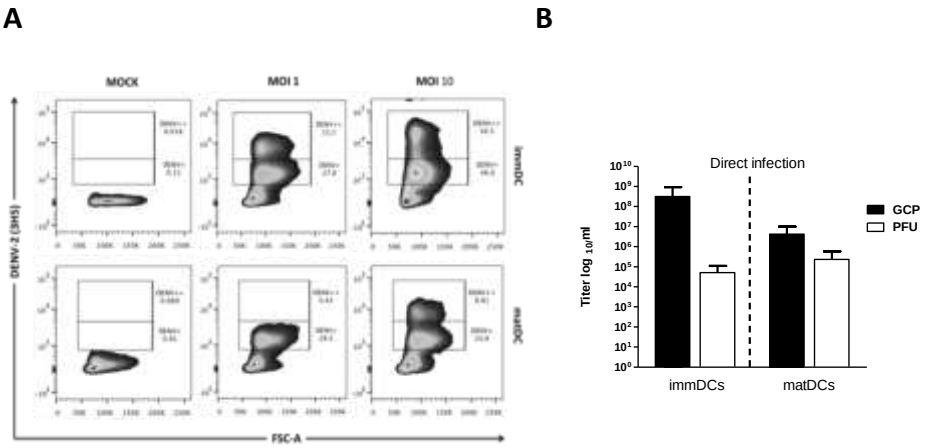

Fig.1. Direct DENV-2 infection in immature and mature DCs. DCs were infected with DENV-2 16681 strain at the multiplicity of infection (MOI) of 1 or 10. At 24 hours post infection cells where fixed and stained intracellularly with an DENV-E- 3H5 mAb; cell-free supernatants were analyzed by qPCR and plaque assay. A) Typical flow cytometric analysis, DENV+ gate denotes DCs that internalized DENV, DENV++ gate comprises DCs actively infected with DENV; (B) number of physical and infectious particles expressed in genome-containing particles (GCP) and plaque forming units (PFU) titers, respectively.

Cells of the innate immune system potently inhibit replication of invading viruses mainly via production of type-I IFN. In fact, DCs are one of the key participants in type-I IFN mediated suppression of virus spread (Scheu *et al.*, 2008). Due to their phagocytic ability, immDCs act as the sentinels of immune system. Phagocytosis of the viral pathogen triggers antiviral responses and drives the transformation of immDCs to antigen presenting matDCs. Paradoxically however, inhibition of viral replication limits the amount of antigen that can be presented to the adaptive immune system. Indeed, it has been recently shown that a specific population of spleen residing macrophages, which filter viral pathogens from the blood stream, produce little IFN I thereby locally allowing non-restricted viral replication (Honke *et al.*, 2011). This mechanism appeared to be crucial for the stimulation of potent adaptive responses as well as increased presentation of antigen to the key IFN I producers such as plasmacytoid DCs (Asselin-Paturel & Trinchieri, 2005).

Interestingly, immDCs produce little to no IFN alpha in response to DENV infection (Mazzon *et al.*, 2009; Munoz-Jordan, 2010; Rodriguez-Madoz *et al*., 2010). Thus, analogically to the abovementioned spleen macrophages, one may expect unrestricted replication in these cells. Yet, we (chapter 5) found that only a small fraction of immDCs support DENV infection. Furthermore, another study showed that DC-derived virus cannot re-infect DCs, which suggests that DCs may not be as important during DENV infection as previously considered (Boonnak *et al.*, 2008; Libraty *et al.*, 2001; Marovich *et al.*, 2001; Nightingale *et al.*, 2008; Palmer *et al.*, 2005). Remarkably, upon close examination of the current literature we noticed that the quality of DENV particles produced by DCs has never been analyzed in detail. The majority of the studies evaluated DENV susceptibility on the basis of intracellular staining of the viral antigens, or by quantification of the physical particles, or infectious particles released (Boonnak *et al.*, 2008; Libraty *et al.*, 2001; Marovich *et al.*, 2001; Nightingale *et al.*, 2008; Palmer *et al.*, 2005); rarely all three measurements have been demonstrated. Considering this lack of knowledge, we herein aimed to re-evaluate the role of DCs as DENV factories during primary and secondary infection.

To this end, we assessed i) the percentage of infected cells; ii) the quality of DENV-2 released by immDCs and matDCs following infection in the absence and presence of potentially enhancing antibody concentrations. ImmDCs and matDCs were cultured and analyzed as described elsewhere (Boonnak *et al.*, 2008; Boonnak *et al.*, 2011), Chapter 5). The cultivated immDCs were characterized as $CD14^{-}$; $HLA\text{-}DR^{low}$, $DC\text{-}SIGN^{high}$ and matDCs were $CD14^{-}$, $HLA\text{-}DR^{high}$, $CD83^{high}$, $CD80^{high}$ $CD40^{high}$. Throughout the study, unless indicated otherwise, the mean of 6 experiments with at least 3 cell donors are shown. Cells were infected with mosquito C6/36 cell-line derived DENV-2 strain 16681 at a multiplicity of infection of 1and 10. At 2 hpi the inoculum was removed and incubation was continued for another 22 hrs. Subsequently, the cells and their supernatants were harvested and analyzed for the frequency of infection and progeny virus production, respectively. The frequency of DENV-infected cells was determined by flow cytometry, as described before (Rodenhuis-Zybert *et al.*, 2010). For staining, DENV-E-specific 3H5 mAb was used. As described in Chapter 5, a unique feature of mAb 3H5 is that it discriminates between antigen internalization (referred to as $DENV^{+}$) and active infection ($DENV^{++}$). As shown in **Fig. 1A**, direct infection (without antibody) led to a higher infection rate in immDCs than in matDCs, 12 % and 16 % vs 0.5 % and 8% of DENV++ cells for MOI 1 and 10, respectively. Surprisingly, however, we observed that many DCs internalize virions but apparently only a limited number of particles is able to escape from degradation and initiates viral replication. At MOI 1, 25-30% of immDC and matDC cultures internalized the virus but failed to induce a productive infection. At high MOI immDCs were more potent in DENV internalization than matDCs, which is in line with the lower endocytotic activity observed in these cells (Boonnak *et al.*, 2008). Next, we examined the quality of the released virus progeny **(Fig.1B)**. Cell-free supernatants from infected immDCs and matDCs were subjected to standard plaque assay on BHK-15 cells (Zybert *et al.*, 2008) for infectious titer determination as well as to qRT-PCR analysis for quantification of the number of genome-containing particles (GCP) (van der Schaar *et al.*, 2007). Surprisingly, despite the marked difference in the frequency of infected cells at MOI 1, we found that both DC phenotypes released the same number of infectious virions (PFU/ml in order of 10^5). Interestingly however, immDCs produced substantially higher levels of viral particles (in order of 10^9 GCP/ml) than matDC (in order of 10^7 GCP/ml), indicating that a large fraction of immDCs-derived virus is non-infectious. Similar results were found

following infection at MOI 0.1 and MOI 10 demonstrating that the effect seen is MOI-independent (data not shown). Thus, DENV-infected immDCs and matDCs, despite the differences in the number of infected cells and secreted physical particles, release an equal number of infectious virions and therefore both cell populations may play an important role in virus dissemination.

The presence of antibodies during natural re-infection is believed to influence DENV infectivity (Boonnak *et al.*, 2008; Endy *et al.*, 2004; Halstead, 2003; Huang *et al.*, 2006). At non- or sub-neutralizing concentrations, pre-existing antibodies are thought to enhance the infected cell mass by facilitating virus cell entry through Fc receptors (Boonnak *et al.*, 2013). To evaluate the role of antibodies on DENV infectivity in the context of DC maturation, we infected the both phenotypes of DCs in the absence and presence of DENV-2 immune-serum. To this end, we first determined the ADE profile of DENV-2 in matDCs.. Therefore in Fig. 2A, a total DENV positive population (DENV+ and DENV++) is shown for both DCs phenotypes. Optimal ADE was observed at a serum dilution of 10^8 and led to a 2- to 3-fold increase in infection rate when compared to direct infection, depending on the donor used. Furthermore, this serum dilution failed to neutralize DENV infection in immDCs (**Fig. 2A**). The increase in infected matDCs mass as compared to direct infection also led to 2- to 4- fold higher virus titers **(Fig. 1B /2B**). Interestingly, in presence of enhancing concentrations of antibody, matDCs produce higher numbers of PFUs than immDCs in the absence and presence of non-neutralizing conditions of antibody. This may suggest that during heterologous secondary infection, matDCs but not immDCs contribute to the increased viral load observed in patients that develop severe dengue.

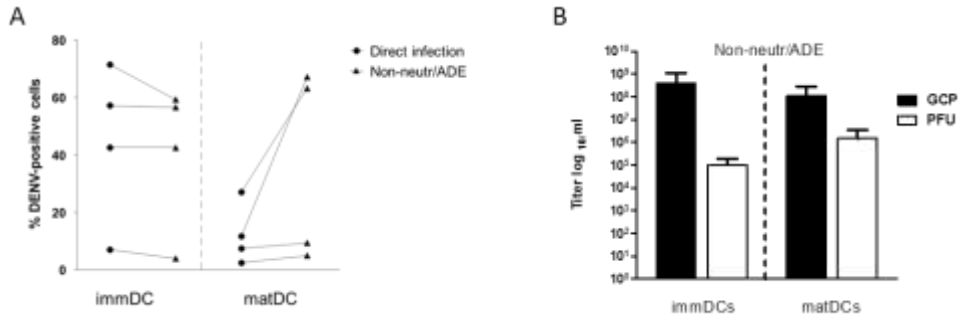

Fig. 2. Effect of non-neutralizing antibody concentration on the susceptibility of DCs to DENV infection. Cells were infected with MOI 1 of DENV-2 alone or DENV-2 pre-incubated with dengue-immune serum at non-neutralizing concentration. A) Frequency of DENV-positive (a sum of DENV+ and DENV++) immDCs and matDCs. Each dot represents average of 3 experiments with 1 donor; B) number of physical and infectious particles expressed in genome-containing particles (GCP) and plaque forming units (PFU) titers, respectively.

To summarize the effects of DC maturation and/or the presence of DENV-antibodies on the quality of the progeny virions, we compared the GCP/PFU ratios of viral particles produced by immDCs and matDCs per individual donor. **Figure 3** clearly demonstrates that the specific infectivity (inverted GCP/PFU ratio) of matDC-derived virus is 10- to 300-fold higher than that of virus released from immDCs. In other words, immDCs although highly permissive to direct DENV infection secrete a large fraction of non-infectious virus particles. Furthermore, the presence of antibody at non-neutralizing concentrations has no substantial effect on the infectious properties of DC-derived virions. Of note, the GCP/PFU ratio of DENV-2 produced in commonly used mosquito and mammalian cell lines varies between 20-100 (Zybert *et al.*, 2008). It is not known why immDCs secrete a large fraction of non-infectious particles. It is however tempting to speculate that specific mechanisms in immDCs exist that support the production of non-infectious particles. Whether the production of poorly infectious virus by immDCs is a phenomenon that is specific to DENV or whether it hallmarks their role as potent innate sentinels remains to be evaluated.

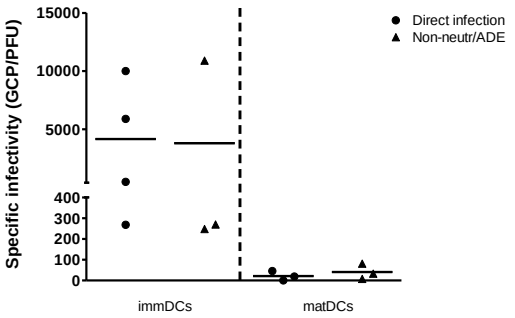

Fig. 3. Specific infectivity of DC-derived DENV. Summary of GCP and PFU titers of DENV-2 released from immDCs and matDCs per analyzed donor.

Secretion of poorly infectious DENV can be controlled by factors associated with the characteristics of the virus and/or cytokines present in the cell supernatant. These include maturation state of the virus, viral envelope glycosylation, and levels of IFN in the supernatant. Furthermore, the cell line used for titration may affect the specific infectivity found. In this study, we have used the same cell to titrate the C6/36- and DC-derived viruses indicating that the observed differences are not related to the cell line used for titration. Furthermore, considering that DENV maturation is more efficient in immDCs than in mosquito-cells (Dejnirattisai *et al.*, 2010) and the lack of IFN I production in immDCs following DENV infection (chapter 5, (Rodriguez-Madoz *et al.*, 2010). We hypothesize that the level of DENV-2 protein glycosylation may play a role in the observed differences in GCP/PFU ratio of immDCs and matDCs-derived DENV. DENV- E protein has 2 potential N-linked glycosylation sites at positions 67 and 153 and both glycosylation sites play important roles in viral infectivity and replication (Mondotte *et al*., 2007). For example, Dejnirattisai et al demonstrated that immDC-derived DENV has a lower infectivity than insect or tumor-cell derived DENV due to changes in viral envelope protein glycosylation (Dejnirattisai *et al.*, 2011). Interestingly, maturation of DCs results in upregulation of N-glycosyltrasferases, enzymes responsible for N-glycosylation of Asn-67 and Asn-153 on DENV- E (Bax *et al.*, 2007) and therefore, increased glycosylation of the viral envelope protein may contribute to the lower GCP/PFU ratio of matDCs-derived virus. We are currently investigating this hypothesis. In summary, herein we showed that immDCs, produce a large fraction of non-infectious virus particles. Conversely, matDCs are less permissive to direct infection but do support enhanced infection in the presence of antibodies. Importantly, matDCs produce highly infectious virus. In conclusion, even though immDCs seem to be a more productive DENV factory than matDCs, the quantity of the product does not always translate to its quality. The ability of matDCs to produce higher numbers of infectious virus in the absence and presence of antibodies suggests that these cells play a more important role in virus dissemination than immDCs.

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Part III

Immature DENV particles: Their contribution in disease severity

Chapter 7

Immature Dengue Virus is Infectious in Human Immature Dendritic Cells Via Interaction with the Receptor Molecule DC-SIGN

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Dengue Virus (DENV) is the most common mosquito-borne infection worldwide. Important target cells during DENV infection are macrophages, monocytes, and immature dendritic cells (immDCs). DENV-infected cells are known to secret a large number of partially immature and fully immature particles alongside mature virions. Fully immature DENV particles are considered non-infectious, but antibodies have been shown to rescue their infectious properties. Therefore, immature DENV particles are thought to only contribute to DENV pathogenesis during secondary infection. In this study, we investigated the infectious properties of fully immature particles on primary immature dendritic cells (imDCs) and a macrophage-like cell line in absence and presence of anti-DENV human serum. We show that immature DENV, although non-infectious in multiple cell lines, exhibits low-level infectivity in imDCs via interaction with DC-SIGN. Furthermore, we demonstrate that imDCs, in contrast to macrophage-like cells, do not support antibody-dependent enhancement of immature DENV. Our data shows that immature DENV can infect imDCs, suggesting that immature DENV particles may contribute to dengue pathogenesis during primary infection. Furthermore, since antibodies do not further stimulate DENV infectivity on imDCs we propose that macrophages/monocytes rather than imDCs are responsible for the increased viral load observed during severe heterotypic DENV re-infections.

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Author summary:

Dengue Virus (DENV) is a mosquito-transmitted virus common in the tropical and sub-tropical regions of the world. There are four serotypes of DENV, and each of these viruses can cause disease ranging from a mild fever to a more severe hemorrhagic fever and hypovolemic shock. Severe forms of disease are often seen during heterotypic secondary infections, due to a complex immunopathological reaction including antibody-dependent enhancement (ADE) of infection. Cells infected with DENV secret virions that vary in maturation state from fully mature to fully immature particles. The latter are considered non-infectious, however several studies have shown that their infectivity can be rescued by anti-DENV antibodies. In this study, we investigated the infectivity of immature DENV in immature dendritic cells (imDCs) and macrophage-like cells, which are known to be important during natural infection. We found that immature DENV exhibits low-level infectivity in imDCs due to interaction with the cell surface receptor DC-SIGN. Furthermore, we show that DENV infectivity cannot be stimulated by antibodies in imDCs. This study expands our knowledge on the role of immature DENV particles and imDCs during primary and secondary DENV infection.

Introduction

Dengue virus (DENV), a flavivirus within the Flaviviridae family, is the most common mosquito-borne viral infectious agent worldwide. According to new estimates, 390 million cases occur annually, of which about 100 million are symptomatic (Bhatt *et al.*, 2013). There are five different serotypes of DENV. Each of them can cause disease ranging from rather mild dengue fever to more severe dengue hemorrhagic fever and dengue shock syndrome (Martina *et al.*, 2009; ScienceInsider, 2013; WHO, 2009). Pre-existing heterotypic antibodies represent a major risk factor for the development of severe disease via antibody-dependent enhancement (ADE) of disease (Halstead, 2003; Halstead, 2007; Kliks *et al.*, 1989). In ADE, pre-existing antibodies are hypothesized to bind to the newly infecting virus serotype and facilitate efficient replication in Fcγ-receptor-expressing cells, thereby increasing the infected cell mass and viral load. A high viral load is often a prelude for severe disease development (Vaughn *et al.*, 2000).

DENV-infected cells secrete a heterogeneous population of virions that vary in maturation state (Cherrier *et al.*, 2009; Junjhon et al., 2008; Junjhon *et al.*, 2010). Earlier studies revealed that the precursor membrane (prM) protein, the hallmark of immature virus particles, caps the envelope (E) protein. Fully immature particles are considered non-infectious, as functional analysis in multiple cell lines indicated that prM affects virus-receptor interaction and membrane fusion activity (Li *et al.*, 2008; Yu *et al.*, 2008; Yu *et al.*, 2009). Indeed, furin-dependent cleavage of prM to M is a prerequisite for membrane fusion activity and infectivity (Elshuber *et al.*, 2003; Elshuber & Mandl, 2005; Moesker *et al.*, 2010; Yu *et al.*, 2008; Zheng *et al.*, 2010; Zybert *et al.*, 2008). Interestingly, antibodies have been found to rescue the infectivity of fully immature DENV particles by Fcγ-receptor-mediated binding and cell entry of DENV-immune complexes. Upon cell entry, the prM protein is cleaved by furin to render the particle infectious (Zheng *et al.*, 2010)(Flipse *et al.*, 2013; Rodenhuis-Zybert *et al.*, 2010). This suggests that fully immature particles are only infectious in presence of antibodies and therefore contribute to the viral load observed in secondary DENV infections.

Immature dendritic cells (imDCs) represent important target cells for DENV replication (Marovich *et al.*, 2001). Virus-cell binding is facilitated through interaction of the glycan moieties that are linked to the DENV's E glycoprotein with the receptor molecule Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) (Tassaneetrithep *et al.*, 2003). Interestingly, a recent report has shown that partially immature particles of West Nile Virus (WNV) – like DENV a member of the flavivirus genus – can infect cells that are engineered to express DC-SIGN (Mukherjee *et al.*, 2011). The glycan moieties on prM were found to interact with DC-SIGN, thereby facilitating virus binding and cell entry. In view of the above studies we here assessed the infectivity of fully immature DENV in primary human monocyte-derived imDCs in the presence and absence of anti-DENV antibodies and compared it with the infectivity of immature DENV in macrophage-like cells.

Materials & Methods

Cell culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by standard density centrifugation using Ficoll-Paque™ Plus (GE Healthcare, Sweden) from buffy coats obtained with written informed consent from healthy, anonymous volunteers, in line with the declaration of Helsinki (Sanquin Bloodbank, Groningen, the Netherlands). Monocytes were isolated by gelatin adherence (Miller *et al.*, 2008) and allowed to differentiate in RPMI (Life Technologies) supplemented with 20% fetal bovine serum (FBS), 500U/ml granulocyte-macrophage colony-stimulating factor (GM-CSF), and 250U/ml recombinant human interleukin-4 (rIL-4) (both from Prospec-Tany, Israel). The medium was replaced every second day till day 6 to generate imDCs. P338D1 cells, a macrophage-like cell line expressing Fcγ-receptors, was maintained in DMEM (PAA Laboratories, Austria) supplemented with 10% FBS, 100U/ml penicillin, 100mg/ml streptomycin, 0.75% sodium bicarbonate (Invitrogen) and 1 mM sodium pyruvate (Gibco) at 37°C / 5% CO_2. Vero-WHO cells were maintained in DMEM supplemented with 5% FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Human adenocarcinoma LoVo cells were maintained in Ham's medium (Life Technologies) supplemented with 20% FBS at 37°C/5% CO_2. C6/36, an *Aedes albopictus* cell line, was maintained in minimal essential medium (Life Technologies) supplemented with 10% FBS, 25 mM HEPES, 7.5% sodium bicarbonate, 100U/ml penicillin, 100mg/ml streptomycin, 200 mM glutamine and 100 mM nonessential amino acids at 30°C/ 5%.

Flow cytometry

The phenotype of the cultured primary imDCs was confirmed at day 6 using flow cytometry analysis, essentially as described before (Boonnak *et al.,* 2011). The cells were analyzed using the primary labeled antibodies Lineage1-FITC, CD11c-APC, HLR-DR-V500, CD80-PE, CD83-PECy7 and CD86-V50 (Becton Dickinson). DC-SIGN levels were determined using an anti-DC-SIGN antibody and a secondary PE-labeled antibody (both R&D systems, MN, USA). Flow cytometry analysis was performed on a LSR-II (Becton Dickinson). Data was analyzed using Kaluza 1.2.

Viruses

For the functional experiments, standard (std) DENV-1 (strain 16007), DENV-2 (strain 16681), DENV-3 (strain H87) and DENV-4 (strain H241), were produced in the *Aedes albopictus* cell line C6/36 essentially as described before (Rodenhuis-Zybert *et al.*, 2010). Briefly, an 80% confluent monolayer of cells was infected at multiplicity of infection (MOI) of 0.1. Depending on the serotype, 72 to 168 hours post infection (hpi), virus was harvested. Immature DENV-1, DENV-2, DENV-3 and DENV-4 were produced in furin-deficient LoVo cells as described (de Alwis *et al.*, 2011). Briefly, an 80% confluent monolayer of cells was infected at MOI 2 and 72 hpi immature DENV was harvested. Preparations for determining specific infectivity were prepared in a similar way, however monolayers were infected with MOI 2 and virus was harvested 72 hours after infection for both immature and std virus and all serotypes.

Infectivity assays

ImDCs were infected at a multiplicity of genome-containing particles (MOG) of 1,000 of either immature DENV-2 or std DENV-2. At 1.5 hpi, fresh medium was added to the cells. The supernatant was harvested at 43 hpi and the number of produced infectious particles was measured by standard plaque assay on BHK-21 clone 15 cells (Zybert *et al.,* 2008). The detection limit of this assay is 20 PFU/ml. The role of DC-SIGN was studied by incubating imDCs 1 h before and during infection with 25µg/ml of either an anti-DC-SIGN antibody or a non-specific isotype control (both R&D systems, MN, USA). To test if viral infectivity could be enhanced by antibodies, immature DENV-2 (MOG 1000) or, as a control, std DENV-2 (MOG 100) was pre-opsonized with 10-fold sequential dilutions of human serum before infection. We used convalescent serum (28 days following infection) from a DENV-2 immune, hospitalized patient (kindly provided by Dr. G. Comach Biomed-UC, Lardidev, Maracay, Venezuela; and Dr. T. Kochel, U.S. Naval Medical Research Center Detachment, Lima, Peru). Infectivity assays on the macrophage-like cell line P388D1 were performed under the same conditions as for imDCs. For antibody-dependent enhancement studies, P3881D1 cells were infected with human serum-opsonized immature DENV-1 and 4 at MOG 1000 or, as a control, non-opsonized std DENV-1 and 4 at MOG 1000.

Quantitative PCR

The number of genome-containing particles (GCPs) was determined by quantitative PCR (qPCR) according to an earlier published protocol based on DENV-2m (van der Schaar *et al.,* 2007). Briefly, the DNA was amplified for 40 cycles of 15s at 95°C and 60s at 55°C (DENV-3) or 60°C (other serotypes). Determination of the number of RNA copies was performed with a standard curve (correlation co-efficient >0.995) of quantified DENV plasmids constructed with standard DNA techniques. For DENV-1: pcDNA3 encoding the M protein sequence of DENV-1 strain 16007; DENV-3: pcDNA3 encoding the E protein sequence of DENV-3 strain 16562; and DENV-4: pcDNA3 encoding the E protein sequence of DENV-4 strain 1036 was used. The details of the primers and probes used for the other DENV serotypes can be found in table S1.

Immunofocus assay

To assess the specific infectivity of immature and std DENV of the different serotypes, the number of infectious particles was determined by an adapted protocol for immunofocus assay (Midgley *et al.,* 2012). One day before titration, 1.3×10^4 Vero-WHO cells were seeded per well in a 96-well plate. Prior to infection, the medium was removed and cells were infected with 10-fold serial dilutions of the virus. After 1.5 h incubation at 37°C, MEM/2% FBS and 1% carboxymethylcellulose (Sigma-Aldrich, Steinheim, Germany) was added as overlay. Cells were fixed and stained after 2 days (DENV-4), 3 days (DENV-2 and DENV-3) or 4 days (DENV-1) of incubation at 37°C/5% CO_2. Prior to the staining procedure, cells were fixed with 10% formaldehyde in phosphate-buffered saline (PBS).

Subsequently, cells were washed with PBS and permeabilized with 2% Triton-X. For detection, 4G2 antibody (Millipore, Temecula, CA) was used as a primary antibody and goat anti-mouse HRP-labeled antibody (Southern Biotech, Birmingham, AL) as a second antibody. Cells were stained with Trueblue Peroxidase Substrate (KPL, Gaithersburg, MD). The foci were counted manually. The limit of detection for immunofocus assay is 20 infectious units (IU) per ml.

Results

Monocyte-derived immature dendritic cells are susceptibility infection of immature DENV

Human primary imDCs were obtained upon culture of PBMC-derived monocytes in the presence of GM-CSF and rIL-4. Six days after culture, the phenotype of the cells was determined by flow cytometry. Figure 1 shows that the cells have a typical imDCs expression pattern: Lin^-, $HLA\text{-}DR^+$, $CD11c^+$, $CD80^-$, $CD83^{low}$, $CD86^{low}$ and DC-SIGN. Importantly, and as expected, imDCs were found to express high levels of DC-SIGN. The

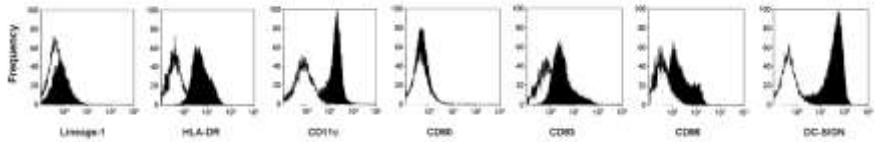

Figure 1. Phenotypic analysis of monocyte-derived immature dendritic cells. Expression profile of different cell type markers by flow cytometry, details are provided in the text. One representative analysis is shown. White curve area: control antibody. Black curve area: specific staining antibody.

differentiated imDCs were infected with a multiplicity of genomeme-containing partcilces (MOG) of 1000 of either immature DENV-2 or std DENV-2. Immature DENV was produced on furin-deficient LoVo cells. The specific infectivity of immature DENV was ~100,000 fold reduced compared to that of std DENV-2, demonstrating that immature DENV-2 is essentially non-infectious in BHK-21-15 cells. The genome-containing particle GCP, as determined by quantitative PCR (van der Schaar *et al.,* 2007) to PFU ratio was 8.4×10^6 for immature DENV compared to 73 for std DENV. Although we never detected infectivity of immature DENV in monocyte and macrophage cell lines or human (da Silva Voorham *et al.,* 2012; Rodenhuis-Zybert *et al.,* 2011), we did observe a low-level infectivity of immature DENV (prM) in imDCs (Figure 2A). At 48 hpi, approximately 1.8×10^3 PFU/ml were produced following infection with immature DENV, while for std virus a titer of 1.4×10^6 PFU/ml was obtained. Similar results were observed in cells of another blood donor (data not shown). Furthermore, to test whether immature DENV infects imDCs due to interaction with DC-SIGN, we treated imDCs with either an anti-DC-SIGN antibody or a non-specific isotype control. Indeed, blockage of the DC-SIGN receptor completely abrogated infection of imDCs with immature DENV (Fig. 2B), indicating that DC-SIGN acts as an entry receptor for immature DENV particles.

Antibody mediated enhancement of immature DENV-2 infectivity on imDCs

The result that immature DENV particles are infectious in imDCs prompted us to also investigate if viral infectivity can be enhanced by anti-DENV serum. Earlier reports performed with std DENV preparations showed that high expression of DC-SIGN is inversely correlated with ADE (Boonnak *et al.,* 2008; Boonnak *et al*., 2011). However, it is not known if this holds true for infection with immature DENV. Therefore, we performed infectivity assays with immature DENV-2 and, as a control, std DENV-2. Before infection, the virus was

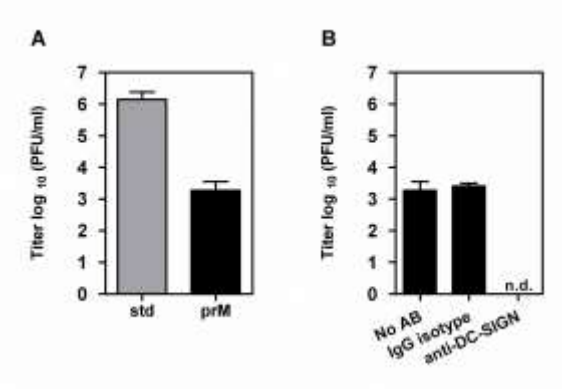

Figure 2. Fully immature DENV-2 particles exhibit basic infectivity on immature dendritic cells. imDCs were infected with MOG 1000 of standard (std) or immature DENV-2 (prM). Supernatant was harvested 43hpi and analyzed. (A) DENV-2 infectivity on imDCs. (B) Role of DC-SIGN on immature DENV-2 infectivity in imDCs as tested by DC-SIGN blockage. Data are expressed as means of at least two independent experiments performed in triplicate; error bars represent standard deviation. N.d. denotes for "not detectable".

pre-opsonized with human serum of a DENV-2 immune individual. As shown in Figure 3A, none of the serum dilutions tested enhanced the basic infectivity of immature DENV in imDCs. Furthermore, and in line with the prior results, no enhancement of std DENV infection was observed (Figure 3B). At lower dilutions ($\leq 10^4$ for immature DENV and $\leq 10^3$ for std DENV), neutralization of viral infectivity was detected. Furthermore, no enhancement of std DENV infectivity was seen upon infection of the cells at lower MOGs (0.1, 1 or 10; data not shown). This shows that the absence of ADE at higher MOG values was not due to saturation of virus particle production capacity in the absence of serum. We achieved comparable results in all donors tested (at least two different donors for each experiment, data not shown), indicating that the outcomes are donor-independent. To test whether the serum possesses inherent enhancing activity, we next performed infectivity assays in P388D1 cells, a macrophage-like cell line expressing Fcγ-receptors. Indeed, and in line with previous published data REF, viral infectivity of immature DENV-2 was enhanced to std virus levels at a serum dilution of $10^4/10^5$. At low serum dilutions, neutralization of infection was observed (Figure 3C and D).

Infectious properties of all DENV serotypes on epithelial cells

Besides testing conditions of homotypic ADE, we pursued to investigate heterotypic enhancement conditions as well. Since the infectious properties of fully immature DENV-1, 3 and 4 have not been described before, we first examined and compared the specific infectivity of both std and fully immature DENV virions of all four serotypes. For this purpose, we analyzed virus particle production on C6/36 cells and LoVo cells 72 hpi by RT-PCR (van der Schaar *et al.*, 2007) and immunofocus assay (Midgley *et al.*, 2012) to measure genome-containing particles and infectious units, respectively. Table 1 shows that immature particles of all four serotypes have a very low specific infectivity on Vero-WHO cells.

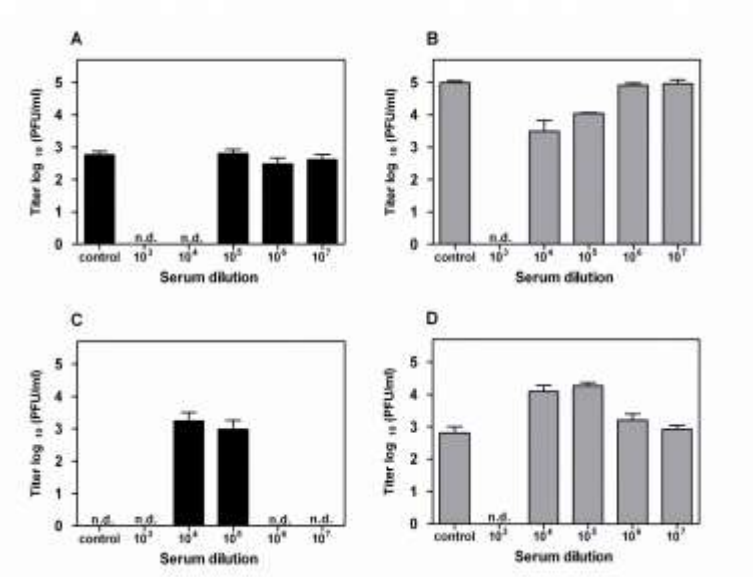

Figure 3. imDCs do not support antibody-dependent enhancement of immature DENV infection. ImDCs were infected with immature DENV-2 at MOG 1000 o 1,000 (A) and std DENV-2 at MOG 100 (B) as mentioned in the text. Data of one representative donor is shown. For each donor, experiments were performed at least in duplicate. P388D1 cells were infected with immature (C) or std (D) DENV-2 at MOG 1000 under similar conditions as in panel A and B. At least two independent experiments were performed in triplicate. Error bars represent standard deviation. N.d. denotes for "not detectable".

When compared to the std DENV preparations, the specific infectivity of immature DENV was about 700-fold reduced for DENV-4 and more than 80000-fold reduced for DENV-2. While the specific infectivity of immature DENV-1 and DENV-3 was at least 2000-fold and 300-fold reduced, respectively. The reduction in specific infectivity may be underestimated for DENV-1 and DENV-3 since we were not able to detect any infectivity (limit of detection of the immunofocus assay is 20 IU/ml).

Antibody mediated enhancement of immature DENV-1, 2 and 4 infectivity on macrophage like cells

Under laboratory conditions, enhancement of DENV infectivity can be detected using homotypic antibodies as long as the antibody concentration is lower than the threshold of neutralization (Beltramello *et al.*, 2010; Boonnak *et al.*, 2011; da Silva Voorham *et al.*, 2012; Rodenhuis-Zybert *et al*., 2010). However, during natural infection, severe disease is predominantly related with heterotypic secondary infection (Halstead, 1988; Halstead, 2007). Therefore, we also aimed to assess the influence of heterotypic human serum on the infectivity of different DENV serotypes. After titrating the virus preparations on Vero-WHO cells, we attempted to analyze the infectivity of DENV-1, DENV-3, and DENV-4 in imDCs. However, using std virus of these serotypes, only a viral output of about 10^3 IU/ml was measured 43 hpi . Unfortunately, the low infectivity of std DENV-1, 3, and 4 in imDCs prevented further characterization of the infectious properties of immature DENV in these cells. In the macrophage cell line P388D1, however, the infectivity of std DENV-1, 3, and 4 was comparable to that of std DENV-2 (figure 3D, control condition) with the another serotypes (figure 4A and B). None of the immature DENV serotypes exhibited infectivity in the absence of serum following infection at MOG 1000. As expected, anti-DENV-2 serum stimulated viral infectivity of immature DENV-1 and DENV-4 to levels comparable to std DENV-1 and std DENV-4 infection (Figure 4A and B). We did not test immature DENV-3, since it was not possible to propagate this virus to

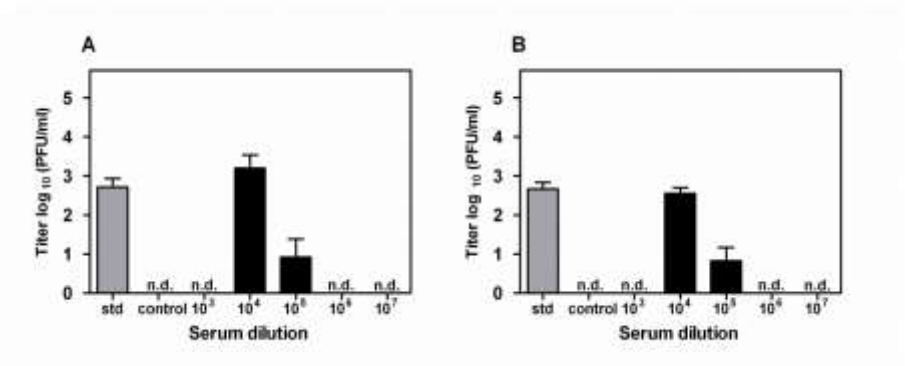

Figure 4. Immature DENV particles of different serotypes can be rendered infectious by heterotypic human serum. P388D1 cells were infected with MOG 1000 of std (grey bars) and pre-opsonized immature (black bars) DENV-1 (A) or DENV-4 (B) as described in the text. At least two independent experiments were performed in triplicate. Error bars represent standard deviation. N.d. denotes for "not detectable".

sufficiently high titers (Table 1). The enhancement profiles were similar for heterotypic and homotypic conditions, which suggests that the antibodies causing ADE of immature DENV are highly cross-reactive.

Discussion

In summary, this study shows that immature DENV particles can infect imDCs via interaction with DC-SIGN. Viral infectivity of immature DENV on these cells is however low and cannot be stimulated by antibodies. In macrophages, antibodies do enhance infectivity of immature DENV, but no differences were found between homotypic and heterotypic serum.

The DENV E glycoprotein is responsible for efficient interaction of the virus with host cells during primary infection. In immature particles, the E protein is obscured by prM, prohibiting efficient virus-receptor interaction (Li *et al*., 2008; Zheng *et al.*, 2010). For this reason, immature particles are presumably scored non-infectious in numerous cell lines, like K562, U937, THP-1, and P388D1, and human PBMCs (Rodenhuis-Zybert *et al.*, 2010; Tassaneetrithep *et al.*, 2003; Zybert *et al*., 2008). However, and in line with recent results with WNV, we here show that immature DENV can bind to DC-SIGN expressed on imDCs. Binding is likely facilitated by sugar groups linked to position Asn^{69} in prM of DENV (Davis *et al.*, 2006; Li *et al.*, 2008; Mukherjee *et al.*, 2011; Yu *et al.*, 2008). Upon binding, the virion enters imDCs via an as yet unknown pathway (Pierson & Diamond, 2012). For partially immature WNV particles, it has been shown that furin cleavage upon entry is not strictly required for WNV infection of DC-SIGN-bearing Raji B cells (Mukherjee *et al.*, 2011). In contrast, several studies have shown that furin cleavage of fully immature DENV or WNV is essential for infection (da Silva Voorham *et al.*, 2012; Rodenhuis-Zybert *et al.*, 2010; Rodenhuis-Zybert *et al.*, 2011). These studies indicate that some but not all prM molecules need to be cleaved upon cell entry to restore viral infectivity. The exact threshold for furin-independent infectivity of partially immature flavivirus particles has not been investigated yet.

The infectious potential of immature DENV in imDCs may imply that these particles contribute - albeit limited - to the total viral load during primary infection. Furthermore, immature particles may be important in the initiation of infection as virus particles produced in mosquito cells are known to have high prM content (Junjhon e*t al.*, 2008; Junjhon *et al.*, 2010). On the other hand, we previously showed that immature WNV particles do not cause disease in mice when injected through the intraperitoneal route (Colpitts *et al.*, 2012; da Silva Voorham *et al.*, 2012). Though, during natural infection, the virus is inoculated in the skin and directly encounters Langerhans cells (Wu *et al*., 2000). These cells express the C-type lectin Langerin, but no DC-SIGN (Geijtenbeek & Gringhuis, 2009). Whether Langerhans cells are permissive to immature DENV infection remains to be elucidated.

Neither std DENV nor immature DENV exhibit ADE on imDCs during heterotypic re-infection. In line with previous observations (Boonnak *et al.*, 2008), we propose that Fcγ-receptors expressed on imDCs do not have an additive effect on viral infectivity due to the high cell surface expression of DC-SIGN. It is, however, possible that

the antibody–opsonized complexes are internalized through the Fcγ-receptor without net increase in viral infectivity. Furthermore, the observation that antibody-opsonized immature DENV has a lower infectivity than std DENV may suggest that immDCs are less efficient in promoting virus maturation upon entry than macrophages. Other target cells like monocyte or macrophage-like cell lines do support enhanced infection of antibody-opsonized immature and std DENV via
Fcγ-receptor-mediated entry, thereby increasing total viral output (Boonnak *et al.*, 2011; Rodenhuis-Zybert *et al.*, 2010). Our results suggest that imDCs generate a similar viral output during primary and secondary heterotypic infection, but do not contribute to the increase of viral load seen in secondary heterotypic infection.

Serotype	**Immature DENV**			**Std DENV**		
	GCP	IU	GCP:IU ratio	GCP	IU	GCP:IU ratio
DENV-1	7.1×10^{7}	N.D.	> 3.6×10^{6} *	8.2×10^{9}	4.8×10^{6}	1698
DENV-2	2.7×10^{9}	217	1.3×10^{7}	2.9×10^{7}	1.6×10^{5}	182
DENV-3	1.7×10^{7}	N.D.	> 8.5×10^{5} *	2.3×10^{8}	1.2×10^{5}	1986
DENV-4	1.8×10^{8}	250	7.3×10^{5}	1.2×10^{10}	1.1×10^{7}	1093

Average results of three independent virus cultures. GCP: Genome containing particles. IU: Infectious units. N.D.: Not detectable. *Based on the detection limit of the immunofocus assay (20 IU/ml).

Acknowledgements

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Chapter 8

Immature dengue virus is a co-factor in disease pathogenesis

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Humoral immunity plays an important role in controlling dengue virus (DENV) infection. Antibodies developed during primary infection protect against sequential infection with the same dengue serotype, but can enhance the disease following heterologous re-infection. Moreover, to evade neutralization of infection DENV is thought to promote inefficient maturation of progeny virions. Indeed, DENV-infected cells are known to secrete particles with varying numbers of precursor membrane proteins (prM) in the viral membrane. Furthermore, we and others observed that the historically regarded non-infectious prM-containing DENV become highly infectious in the presence of E- and prM-antibodies. Accordingly, we hypothesized that these virions can contribute to the exacerbation of disease during heterologous re-infection. To this end, we here investigated the ability of acute sera of 30 DENV-2 infected patients with different grades of disease severity, to bind, neutralize and/or enhance immature DENV-2 infection. We found that a large fraction of antibodies bind to immature DENV-2, but there is no significant difference between the disease severity groups. Furthermore, functional analysis of the antibodies did not underscore any specific correlation between the neutralizing/enhancing activity towards immature DENV-2 and the development of more severe disease. Based on our current results, we conclude that immature virions are not a discriminating factor but rather act as a co-factor in dengue pathogenesis.

Manuscript in preparation

Introduction

Dengue is the most prevalent mosquito-borne viral disease worldwide. Each year, 50-100 million infections occur mainly in the (sub) tropical regions of the world (Mackenzie, Gubler, & Petersen, 2004). Symptomatic infection with any of the four DENV serotypes can manifest as dengue fever (DF), a self-limiting febrile illness or lead to the more severe and potentially life-threatening dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS) (WHO, 1997). Pathogenesis of severe DENV infection is multifaceted (Rodenhuis-Zybert, Wilschut, & Smit, 2010), but sequential infection with a heterologous DENV strain as well as primary infection of infants with declining levels of maternal antibodies are considered major risk factors for severe disease. Accordingly, it has been hypothesized that antibodies generated during primary infection or passively acquired through DENV-immune mothers can exacerbate disease via so-called antibody-dependent enhancement (ADE) of infection. In ADE, cross-reactive, non-neutralizing antibodies are thought to facilitate successful entry of virus-immune complexes in Fc-receptor-bearing cells (Burke & Kliks, 2006; Halstead & O'Rourke, 1977; Kliks *et al.*,1989) ultimately leading to an increased viral load early in infection– one of the hallmarks of severe disease development (Balsitis *et al*., 2010). In vitro, ADE of DENV infection can be observed in many Fc-receptor-expressing cell lines, including K562, U937, P388D1 and in primary human cells like monocytes, macrophages and mature dendritic cells (Halstead & O'Rourke, 1977; Morens *et al.*,1987).

The viral surface of mature DENV is covered with 180 copies of 2 transmembrane glycoproteins: the membrane (M) and envelope (E) protein. The ectodomain of the E protein has three structurally distinct domains (DI, DII, DIII) and is responsible for the infectious cell entry of the virion (Modis *et al.*, 2003; Nybakken *et al.*, 2006; Rey *et al.*,1995; Y. Zhang *et al.*,2007). Immature virions, primary products of viral assembly, are structurally distinct from mature particles and contain 180 heterodimers of the E and precursor (pr)M protein in the viral membrane. Immature DENV matures during transit through the Golgi and trans-Golgi network, where the cellular protease furin cleaves prM to M and a "pr" peptide. The "pr" peptide is released upon secretion of the particle into the pH neutral extracellular milieu (Li *et al.*, 2008). The prM protein inhibits the fusogenic activity of the E protein thereby protecting newly assembled virions from premature fusion within the mildly acidic compartments of the exocytotic pathway (Li *et al.*, 2008). Importantly, the maturation process of DENV appears to be inefficient as infected cells release significant numbers of immature and partially mature virions. In fact, around 30-40% of the particles produced in mosquito cells still contain uncleaved prM protein (Junjhon *et al*
., 2008; Zybert *et al.*,2008).

The E protein is the principal target of neutralizing and enhancing antibodies developed in response to DENV infection (Beltramello *et al.*, 2010; Dejnirattisai *et al.*, 2010; Lai *et al*., 2008). The majority of antibodies are raised against EDII domain and in vitro analysis revealed that these antibodies often have weakly neutralizing properties and a high degree of serotype cross-reactivity (Beltramello *et al.*, 2010; Crill *et al.*,2009; Lai *et al.*, 2008). Only a small fraction of the total specific humoral response is directed against EDIII domain and these mostly represent potent neutralizing serotype-specific antibodies (Beltramello *et al.*, 2010; Crill *et al.*, 2009). Furthermore, and in line with the incomplete maturation status of standard (std) DENV preparations in vitro, prM-antibodies are generated during primary and secondary DENV infections (de Alwis *et al.*, 2011; Dejnirattisai *et al.*, 2010). Notably, several studies reported significantly higher levels of prM antibodies in secondary infection when compared to primary infections (Lai *et al.*, 2008; Valdes *et al*., 2000). Antibodies directed against prM generally appear to poses non- or weakly neutralizing properties and are highly cross-reactive between DENV serotypes (Beltramello *et al.*, 2010; de Alwis *et al.*, 2011; Dejnirattisai *et al.*, 2010). We and others have shown that in the presence of prM or E antibodies, the historically regarded non-infectious immature DENV particles become highly infectious (Chan *et al.*, 2012; da Silva Voorham *et al.*,2012; Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*,2010; Rodenhuis-Zybert *et al.*,,2011). Antibody-opsonized immature DENV binds to Fc-receptors and this interaction facilitates cell entry of the immune complex via an as yet unknown pathway. The acidic environment of endosomes/phagosomes subsequently triggers conformational changes in the virion allowing furin to cleave the prM protein (Rodenhuis-Zybert *et al.*,2010). Furthermore, the low pH conditions in the late endocytic/phagocytic pathway (to around 5.0) is thought to prompt the release of "pr" peptide thereby rescuing the fusion and infectious capacity of the virion (X. Zhang *et al.*, 2003). Accordingly, in presence of antibodies, immature particles can expand the pool of infectious virus thereby likely contributing to the DENV pathogenesis (Chan *et al.*, 2012; da Silva Voorham *et al.*,2012; Dejnirattisai *et al.*, 2010; Rodenhuis-Zybert *et al.*,2010; Rodenhuis-Zybert et al.,2011).

In this study we investigated the potential role of immature DENV in disease pathogenesis. First, we examined the antigen-specificity in patients with acute DENV-2 infection and compared the prM to E antibody ratio per disease severity (DF, DHF, DSS). Next, we tested the capacity of the sera to neutralize and/or enhance the current infecting

DENV serotype. We show that prM-antibodies were present in all immune sera tested. Moreover, immune sera from all severity groups were able to bind to and enhance infectivity of immature DENV-2 particles. Notably, antibody-mediated enhancement of std DENV-2 infection by the sera partially relied on enzymatic activity of furin. Counter intuitively however, we found no significant difference between the 3 disease groups. This suggests that antibodies recognizing immature DENV cannot be used as a predisposing factor for severe disease development. Immature DENV particles act as a co-factor in dengue pathogeneses by enhancing the number of infectious virions circulating during secondary infection.

Results

A significant fraction of human antibodies is directed against the prM protein

First, we investigated if the prM antibody response is more abundant in patients with severe disease. To this end, we loaded immature DENV-2 virions on a SDS-PAGE gel and performed Western blot (WB) analysis using acute secondary DENV-2 sera. To rule out individual bias we pooled sera of 10 patients per disease group by triplicate. The specific antibody response and the ratio of prM and E antibodies was quantified by means of ImageQuant TL software, as described in Materials and Methods. The total prM and E antibody response was comparable between DF, DHF, and DSS patients (data not shown). Approximately 20-35% of the antibody response was directed to the prM protein (Fig. 1). The prM antibody response was slightly higher in the DF group albeit not statistically significant, indicating that a prM antibody response is not indicative for severe disease development.

Dengue specific antibodies recognize immature virions

Recent work showed that most of the antibodies generated against prM and E are conformation sensitive (Lai *et al*., 2008; Roehrig *et al*.,1998), implying that the identification of certain IgG clones can be underestimated by means of WB technique (de Alwis *et al.,* 2011). To rule out this possibility, we next analyzed the capacity of the human IgGs to react with immature and std DENV-2 preparation by means of indirect ELISA, as described in Material and Methods. Figure 2 depicts representative reciprocal end-point titers obtained from at least three independent experiments. Note that immune sera from all three disease severity groups contain antibodies that bind to immature (Fig. 2A) and std DENV-2 (Fig. 2B). Importantly however, in line with the WB results, we did not detect significant difference in the relative binding of immature and std DENV between the severity groups.

Dengue-immune sera promotes infectivity of immature DENV-2 in a furin-dependent manner

Having established that the quantity of immature-virus specific antibodies does not distinguish disease severity, we next evaluated the quality of these responses. We tested the capacity of the acute sera to neutralize and/or enhance DENV infection using the DENV serotype causing current infection. Viral infectivity was measured in murine macrophage-like P388D1 cells, which express three distinct Fc-γ-receptors (FcγR), FcγRIII [CD16], FcγRII [CD32], and FcγRI [CD64]) (Ochiai *et al.,*1988; Sung *et al.,* 1985). Cells were infected with immature and std DENV-2 at MOG 500 in the absence or presence of increasing dilutions of pooled immune sera. After 1 round of infection, we harvested the supernatant and analyzed the infectious virus production. As shown in Figure 3, immune sera of all severity groups neutralized immature DENV-2 particles at a dilution of 400x or lower. Enhancement of infectivity was observed at higher dilutions 1600x-102400x (Fig. 3A, B and C). Importantly, at most enhancing conditions, the titers of immature DENV-immune complexes reached comparable levels as std DENV-2 in the absence of immune sera. These findings are consistent with previous studies, which demonstrated that immature DENV particles are infectious in the presence of polyclonal sera, anti-prM and anti-E (da Silva Voorham *et al*.,2012; Dejnirattisai *et al.*, 2010; Ochiai *et al.,* 1988; Rodenhuis-Zybert *et al*.,2010; Rodenhuis-Zybert et al.,2011). Furthermore, and as expected, immune sera from all severity groups enhanced std DENV-2 infection (Fig. 3D, E and F), albeit with a lower power of enhancement when compared to that of immature particles. Interestingly, DHF sera was most efficient in neutralizing std DENV-2 infectivity at high sera concentrations and elicited significant ADE over a broadest range of sera dilutions (Fig. 3E).

We previously demonstrated that antibody-opsonized immature viruses require enzymatic activity of furin in host cells to become infectious (da Silva Voorham *et al*.,2012; Dejnirattisai *et al.*, 2010; Ochiai *et al.*, 1988; Rodenhuis-Zybert *et al*.,2010; Rodenhuis-Zybert et al.,2011). To investigate whether furin activity is also critical for the infectivity of immature particles opsonized with polyclonal sera,

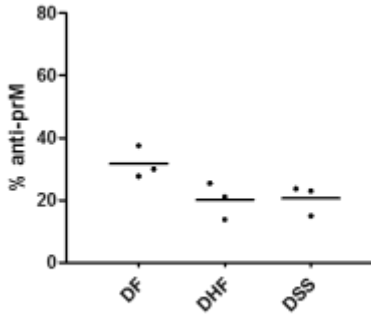

Figure 1. Specificity of human antibody response against DENV prM protein. Western Blot analysis of pooled immune sera from patients with DF, DHF and DSS binding to purified immature DENV-2. Student's t-tests were used to determine significance, no significant difference was observed.

we inhibited activity of cellular furin in P388D1 cells using a specific furin inhibitor decRRVKR-CMK (FI), as described previously (Rodenhuis-Zybert *et al.*, 2010). Under the conditions used, FI did not affect maturation of newly assembled virions (control bars in Fig. 4A and 4B). Importantly, inhibition of furin activity in target cells abolished infectivity of immature virions opsonized with immune sera. These results substantiate our previous findings indicating that maturation upon entry is a prerequisite for rendering DENV-immune complexes infectious (Fig. 4A).

ADE of std DENV-2 elicited by immune sera is partly dependent on furin activity

The circulation of prM antibodies in patients sera is believed to mirror the existence of prM-containing (partially) immature virions in vitro DENV preparations. Despite our limited understanding on the functional role of (partially) immature virions, it has been speculated that viral infectivity is inversely related to prM content (Rodenhuis-Zybert *et al.*, 2011). Particles that have a high prM content being essentially non-infectious in multiple cells (Zybert *et al.*, 2008 da Silva Voorham *et al.*, 2012; Rodenhuis-Zybert, *et al.*, 2010). Interference with furin activity can therefore tell us which proportion of the ADE observed during std DENV infection is caused by immature particles that require maturation upon cell entry. Indeed, we previously reported up to 10-fold reduction in viral infectivity following infection of mAb opsonized-std DENV-2 in FI-treated cells (da Silva Voorham2012; Rodenhuis-Zybert *et al.*,2011). To assess the extent to which (partially) immature virions contribute to ADE in std DENV2 preparations, cells were treated with FI and tested for their ability to cause ADE of DENV2 opsonized with sera from DF, DHF and DSS patients. The sera dilution that gave the best ADE in non-treated cells was used for this experiment. Figure 4B shows a significant reduction in viral infectivity in cells treated with FI, strengthening the hypothesis that ADE is also caused by (partially) immature particles present within standard virus preparatio

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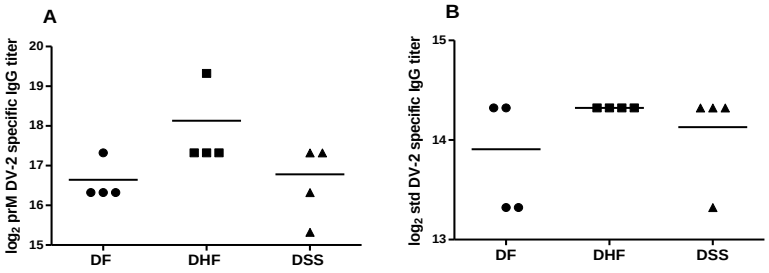

Figure 2. Capacity of immune sera to bind immature and std DENV-2. Presence of IgGs directed binding to Immature DENV-2 (A) and std DENV-2 (B) preparations were analyzed by means of indirect ELISA. Each dot represent reciprocal end point titer obtained in one of at least three independent experiments. The horizontal lines depict average of all experiments. No significant difference was found between the groups.

Discussion

In this study we investigated the potential involvement of immature DENV particles in dengue pathogenesis. We tested the ability of acute DENV-2 sera obtained from DF, DHF and DSS patients to bind and enhance infectivity of immature DENV-2. We found that a substantial fraction of antibodies is directed against the prM protein and immature virions, however no significant difference was observed between the distinct severity groups. Furthermore, similar ADE profiles were obtained following infection with immature virions pre-opsonized with sera from DF/DHF/DSS patients, suggesting that the presence of immature particles and the antibodies recognizing them cannot be seen as a predisposing factor for severe disease. Nevertheless, we demonstrated that prM-containing virions within std DENV-2 preparations significantly contribute to ADE, indicating that immature particles act as a co-factor in disease pathogenesis. Only a small fraction of the humoral response elicited during DENV infection appears to be responsible for protection (Beltramello *et al*., 2010; Crill *et al.*, 2009; de Alwis *et al*., 2011; Dejnirattisai *et al.*, 2010; Lai *et al.*, 2008). The great majority of dengue specific antibodies being cross-reactive and weakly neutralizing. Accordingly these antibodies are thought to exacerbate disease during heterologous re-infection. In line with earlier observations (Beltramello *et al.*, 2010; de Alwis *et al*., 2011; Dejnirattisai *et al.*, 2010), we found that a large fraction of antibodies are produced against the prM protein. Furthermore, and in agreement with Lai and co-workers, there was no significant difference in prM antibody levels between DF, DHF, DSS patients. This indicates that there is no direct correlation between the level of prM antibodies early in infection and subsequent disease presentation. Since antibodies directed towards E can also

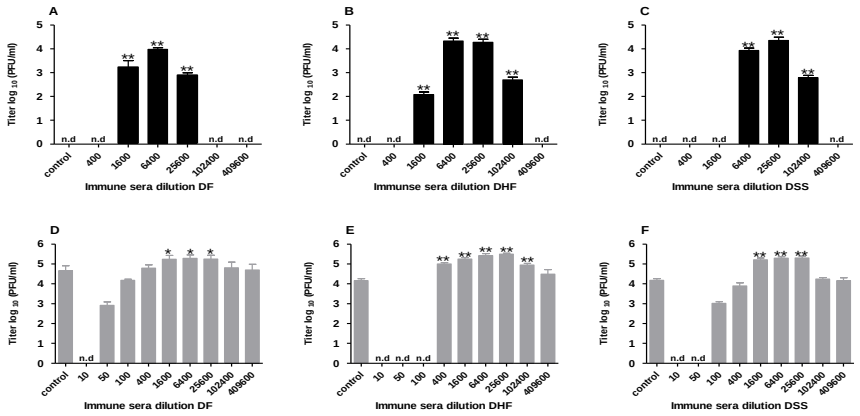

Figure 3. Immune sera neutralizes and enhances the immature and std DENV-2 infection. P388D1 cells were infected with immature or std DENV-2 at MOG 500 in the presence or absence of serially diluted pooled-sera of DF patients (A and D), DHF (B and E) patients and DSS patients (C and F). At 43 hpi supernatant was harvested and virus production was analyzed by plaque assay on BHK21-15 cells. Data are expressed as means of at least two independent experiments performed in triplicate. The error bars represent standard deviations (SD); (n.d.) denotes ''not detectable''. Student's t-tests were used to determine significance; *, *P* < 0.05; **, P < 0.01

bind and enhance infectivity of immature virions (da Silva Voorham *et al.*,2012; Rodenhuis-Zybert *et al.*,2010), we also studied the capacity of immune sera to react with immature virions by means of ELISA. We demonstrated that an important fraction of the antibodies produced during natural infection is able to bind to immature particles; however

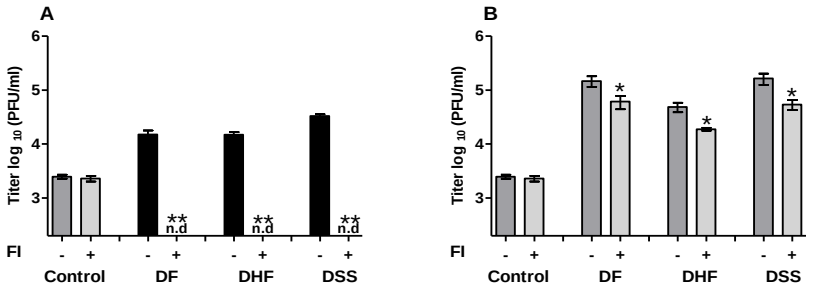

Figure 4. ADE of immature and std DENV-2 infection by immune sera can be influenced by furin activity. Non-treated P388D1 cells and the cells treated and with FI were infected with immature (A) and std (B) DENV-2 strain 16681 at MOG 500 in the absence or presence of pooled immune sera. Virus production was detected as described in the legend of Fig.1. The error bars represent standard deviation (SD) derived from at least two separate experiments performed in triplicate. Two-tailed Student's t-tests were used to determine significance; *, *P* < 0.05; **, P < 0.01.

the presence of such antibodies is not discriminative for severe disease development. Taken together, these findings suggest that antibodies recognizing immature virions cannot be used as a diagnostic tool to investigate if a patient is predisposed to severe disease. In vitro, DENV preparations comprises a mixture of mature, partially and fully immature particles (Junjhon *et al*., 2010). Importantly, the extent of virion maturation influences the capacity of antibodies to neutralize or enhance infection (Nelson *et al*., 2008). Accordingly, it has been speculated that "inefficient" cleavage of prM can serve as an immune evasion mechanism. This notion is supported by recent observations indicating that most prM and E antibodies that preferentially bind to immature virions are cross-reactive and weakly or non-neutralizing (Dejnirattisai *et al*., 2010). An important remaining question is, how many prM-containing particles circulate in DENV-infected humans. We have attempted to answer this question by combinatory antigen capture ELISA/qPCR assay. Unfortunately, despite high viremia titers in acute sera of the patients, the numbers of ELISA captured virions were insufficient to reach the threshold necessary for reliable qPCR measurements. Instead we analyzed the overall contribution of (partially) immature virions to the ADE profile. We took advantage of the fact that fully and, probably also partially immature virions with a high prM content rely on enzymatic furin activity to become infectious (Mukherjee *et al*.,2011; Rodenhuis-Zybert *et al*.,2011; Rodenhuis-Zybert,2011). Although no differences were found between disease severity groups, (partially) immature virions present within std DENV preparations significantly contributed to the overall ADE effect observed. These results strongly suggest that immature particles that depend on endosomal furin cleavage contribute to the total viral load observed during secondary dengue infection. Whether the remaining, furin-independent, ADE of infection is caused by partially immature virions or the fully mature particles remains to be elucidated. Multiple studies (Dejnirattisai *et al*., 2010; Huang *et al*., 2006; Rodenhuis-Zybert *et al*., 2010) show that prM antibodies are more prone to initiate ADE rather than neutralization. Therefore one could argue that prM antibodies are detrimental for protection and should not be elicited by a DENV vaccine. Unexpectedly, although though no significant results were obtained between disease severity groups, sera from DF patients had relatively more prM antibodies and had a narrow spectrum of antibody neutralization. Moreover, sera of DHF patients neutralized the infecting serotype over the broadest range of dilutions. Noteworthy in this respect are the recent results from the Sanofi trail indicating that high antibody titers are not indicative for DENV-2 protection (Sabchareon *et al*., 2012). Clearly, these results show that we do not yet understand the correlate of protection against DENV disease. It is therefore imperative to better understand the immune response in vaccinated individuals and it would be of interest to determine the ratio of prM and E antibodies in this cohort. The unforeseen and disappointing results of the clinical trial indicate that more fundamental research is required to unravel which antibodies are most protective and should be elicited by a vaccine. What then determines the development of severe disease? At present, we cannot answer this question but it is clear from this and many other studies that dengue virus pathogenesis is very complex and involves multiple virus and host factors (Halstead, 2007; Martina *et al*., 2009; Rodenhuis-Zybert *et al*., 2010). The infection history of the patient in combination with the current infecting DENV genotype will determine the balance between neutralizing and enhancing antibodies. These together with yet unspecified host factors will likely predispose to more severe

disease. Evidently, it remains an enormous challenge to fully understand why some individuals develop severe disease whereas others do not.

Materials and Methods

Patients.

Immune sera was obtained from the ongoing Pediatric Dengue Cohort Study in Nicaragua. DF, DHF and DSS were diagnosed according to WHO case definitions (WHO, 1997). All patients had an acute secondary DENV-2 infection, defined by an antibody titer by inhibition ELISA ≥ 20 (equivalent to an HI titer ≥ 10) (Harris *et al.*, 2000; Nogueira *et al.*, 1993). The sera used in this study was taken 2 to 4 days after the onset of fever. In total 30 patients: 10 with DF, 10 with DHF and 10 with DSS were analyzed. For analysis, the 10 samples per disease severity were pooled.

Cells.

Aedes albopictus C6/36 cells were maintained in minimal essential medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPES, 7.5 % sodium bicarbonate, penicillin (100 U/ml), streptomycin (100 µg/ml), 200 mM glutamine and 100 µM nonessential amino acids at 30°C, 5 % CO_2. Baby hamster Kidney clone 15 cells (BHK21-15) cells were cultured in DMEM (Life Technologies) containing 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), 10 mM HEPES, and 200 mM glutamine. Human adenocarcinoma LoVo cells were cultured in Ham's medium (Invitrogen) supplemented with 20% FBS at 37°C, 5% CO_2. Mouse macrophage P388D1 cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml), sodium bicarbonate (Invitrogen, 7,5% solution) and 1.0 mM sodium pyruvate (GIBCO) at 37°C, 5% CO_2.

Virus growth.

DENV-2 strain 16681 was propagated on C6/36 cells, as described before (Zybert *et al.*, 2008) Immature DENV-2 was produced on LoVo cells, as described previously (Zybert *et al.*, 2008). Briefly, LoVo cells were infected at MOI 5, 1.5 h post-infection (hpi) the virus inoculum was removed, cells were washed three times with PBS, and fresh medium was added. At 72 hpi, the medium containing the virus particles was harvested, cleared from cellular debris by low-speed centrifugation, aliquoted, and stored at -80ºC. The specific infectivity was determined by measuring the number of infectious units by plaque assay on BHK21-15 cells and the number of GCPs by quantitative PCR (qPCR) analysis, as described previously (van der Schaar *et al*., 2007).

Western Blot.

For Western blot analysis, $1.0x10^9$ GCPs of purified immature DENV-2 were loaded on 12.5% SDS polyacryramide gels under non-reducing conditions. The blot was incubated with serial dilutions of pooled immune sera from DF, DHF and DSS patients. A human monoclonal prM antibody hmAb 27.2 (kind gift from A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) was used as a positive control. The antibody response against the E and prM protein was analyzed with ImageQuant TL. The percentage of prM is calculated by relating the intensity of the prM protein band to the total intensity of prM and E. Values obtained from three independent experiments were compared by Student's t-test and ANOVA.

ELISA

The binding properties of dengue-immune sera to immature virus particles was assessed with a three-layer ELISA. Briefly, microtiter ELISA plates (Greiner bio-one) were coated overnight with $1x10^8$ GCPs of purified virus per well in 100 µl coating buffer. After blocking with 2% milk in coating buffer for 120 min, 100 µl of two-fold serial dilutions of control and pooled dengue-immune sera from patients with DF, DHF and DSS were applied to the wells and incubated for 1.5 h. Subsequently, upon extensive washing, 100 µl of horseradish peroxidase-conjugated mouse anti-human IgG antibody (Southern Biotech 9040-05) was applied for 1 h. All incubations were performed at 37°C. Staining was performed using o-phenylene-diamine (OPD) (Eastman Kodak Company) and absorbance

was read at 492 nm (A_{492}) with an ELISA reader (Bio-tek Instruments, Inc.). The highest positive (cut off = mean control sample OD + 3xSTDEV of control) in the assay was defined as the endpoint titer.

Infectivity assays.

Virus or virus-immune sera complexes were added to a monolayer of P388D1 cells (2×10^5) in 24 wells plates (Costar), at a multiplicity of genome-containing particles (MOG) per cell of 500. At 1.5 hpi, fresh medium was added to the cells. At 43 hpi, the medium was harvested and virus production was analyzed by plaque assay on BHK21-15 cells, as described previously (Diamond & Harris, 2001). The limit of detection in the plaque assay is 20 PFU/ml. In furin inhibitor experiments, cells were treated with 25 µM of furin-specific inhibitor, decanoyl-L-arginyl-L-valyl-L-lysyl-L-arginyl-chloromethylketone (decRRVKR-CMK) (Calbiochem) prior to and during virus infection (Rodenhuis-Zybert *et al.*, 2010).

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Part IV

Summarizing discussion

Chapter 9

Summarizing discussion and concluding remarks

Summary

Dengue is the most prevalent mosquito-borne viral disease in the world. Previously dengue was confined to tropical regions but in recent years it has spread to areas with sub-tropical and moderate climates (Hayes *et al.*, 2006; Moi *et al.*, 2010; WHO, 1997). In fact, in 2012 the first dengue outbreak was observed within Europe (Sousa *et al.*, 2012; WHO, 2013). An estimated 400 million infections occur annually (Bhatt *et al.*, 2013). Infection with dengue virus (DENV) can induce multiple clinical manifestations ranging from a self-limited fever called dengue fever (DF) to life-threatening manifestations, such as dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) (WHO, 1997). There are 5 antigenically distinct serotypes (DENV-1 to DENV-5), the fifth one was only recently discovered in Malaysia (ScienceInsider, 2013).

All DENV serotypes are human pathogens. The immune response generated during a primary DENV infection appears to have a dual role upon re-infection (van der Schaar *et al.*, 2009). During homotypic re-infection it controls viral infection and no disease symptoms develop. In approximately 2% to 4% of the individuals re-infected with a heterologous DENV serotype, however, the immune response is believed to underlie the development of severe disease. Indeed, exacerbation of disease during heterologous re-infection has been linked to the phenomenon of original antigenic sin of T cells and antibodies (Halstead & O'Rourke, 1977; Halstead, 2003; Mongkolsapaya *et al.*, 2003). This implies that the antibody and T cell response is skewed towards the primary infecting DENV serotype and as a consequence high numbers of cross-reactive antibodies and low avidity T cells are generated during secondary infection. Cross-reactive antibodies have been shown to facilitate enhancement of infection thereby increasing the infected cell mass and viral burden. Imbalanced secretion of cytokines by T lymphocytes subsequently increases vascular permeability, which appears to be the principal factor that gives rise to the development of severe disease. Pre-existing antibodies appear to represent an essential trigger as infants, with waning maternal dengue antibodies have an increased risk to develop severe disease during primary infection (Simmons *et al.*, 2007).

During primary DENV infection, the three major phagocytic cell types of the innate immune system, monocytes, macrophages, and dendritic cells (DCs) have been found to be preferentially infected (Blackley *et al.*, 2007; Marovich *et al.*, 2001; Wu *et al.*, 2000). During secondary infection, antibodies from a prior infection facilitate viral entry into Fc receptor (FcR) bearing cells, such as monocytes, macrophages, and DCs. In case of a homotypic re-infection, most virus-antibody complexes will be degraded upon FcR-mediated phagocytosis (Heusser *et al.*, 1977). In case of a heterologous re-infection leading to severe disease, the virus-antibody complexes escape from the degradative pathway and induce a productive infection. Thus, during primary and secondary infection, immune cells are the predominant infecting cell types. Infection of immune cells by DENV may significantly impact the development of the immune response, because these cells are essential for the initiation and polarization of adaptive immune response. To recognize the intruders, these cells are equipped with a set of phylogenetically conserved pattern recognition receptors (PRRs), such as Toll-like Receptors (TLRs) [**revised in chapter 2**] (Torres-Pedraza *et al.*, 2010). TLRs recognize pathogen-associated molecular patterns (PAMPs) and danger associated molecular patterns (DAMPs) and in DCs, their activation leads to a positive regulation of co-stimulatory molecules, HLA molecules, cytokines, chemokines, and other soluble mediators. These signals are required to establish a cellular immune response (Banchereau & Steinman, 1998) and an antiviral state. However, many viruses, including DENV, exert mechanisms that aim to evade innate signaling and sustain an efficient DENV replication [**revised in chapter 1**]. The characterization of these early events that lead to either protection or the virus spreading is essential to understand dengue pathogenesis and is the central focus of this thesis.

In **chapter 4**, we explored the activation of TLRs in monocytes, myeloid, and plasmacytoid DCs (mDCs and pDCs, respectively) from individuals who developed different manifestations of the disease. We found differences in TLRs expression and activation status of DCs from DF and DHF patients. A higher expression of TLR2 in pDCs was observed in individuals with DHF when compared to DF patients. In addition, we found that mDCs from DHF patients had a significant lower expression of TLR3 and TLR9 compared to DF patients. Furthermore, DCs from patients with hemorrhagic manifestations expressed lower levels of the maturation markers CD80 and CD86 than DF patients. These results show that during dengue disease progression, the expression profile of TLRs changes depending on the severity of the disease. Alteration in TLRs signaling could also directly affect DC activation, thereby influencing the innate immune response. In order to validate our *ex vivo* study, we also assessed TLRs functionality *in vitro*. We evaluated the capability of DENV infected peripheral blood mononuclear cells to produce IFN in response to the stimuli and in presence of several TLRs agonists. We found that there was a poor stimulation of IFN production via TLR9, suggesting that DENV alters IFN response through this signaling pathway.

In chapter 5, we characterized the phenotypical and functional changes that immDCs undergo after DENV infection in the absence or the presence of antibodies. We demonstrated that direct DENV infection - unaided by antibodies - blocked maturation and cytokine production of immDCs. ImmDCs directly infected with DENV did not up-regulate the expression of maturations makers such as CD83, CD86 and HLA-DR. In contrast, we observed that exposure of DCs with DENV opsonized with neutralizing concentrations of antibodies stimulated full DC maturation and triggered a cytokine response. At these conditions we found a balanced pro- inflammatory (IL-6 and TNF-alpha) and anti-inflammatory (IL-4, and IL-10) response. The increased cytokine production was strictly dependent on the interaction of the immune-complex with FcγRIIa. Interestingly, despite a similar level of infection was seen following infection at non-neutralizing serum conditions and direct infection (virus unaided to antibodies), only non-neutralizing conditions lead to an increase in HLA-DR expression and cytokine production. These findings show that the presence of immune-complexes modulate the signaling pathways that lead to activation of DCs.

In order to expand our knowledge on the role of DCs in DENV dissemination [**chapter 6**], we evaluated the infectious properties of the particles produced by immature and mature DCs in the absence and presence of DENV-immune serum. A higher specific infectivity of the viral progenies generated by matDCs was found when compared to the particles produced by immDCs. We found that the frequency of DENV positive cells in immDCs was approximately 3 times higher than DENV positive cells in matDCs. This high susceptibility of immDCs to DENV infection can be explained by its high content of the dendritic-dells specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) receptor (Boonnak *et al.,* 2008; Sun *et al.,* 2009). We also infected immDCs and matDCs in the presence of a DENV-2 immune-serum. Our results reveal that DENV-immune serum enhances viral infection in matDCs but not in immDCs. Taken together, the findings from **chapters 5 and 6** suggest that, even though immDCs are slightly more permissive to DENV infection than matDCs, their contribution to virus dissemination might not be as significant as initially thought. In fact, immDCs and matDCS may be equally important during primary DENV infection. During secondary heterotypic infection, matDCs are likely to become the main viral factories contributing to increased viremia that often preludes disease exacerbation.

Furthermore, the statement that DENV-infected cells produce heterogeneous populations of virions that vary in their maturation state prompted us to investigate whether immDCs are susceptible to immature DENV particles. The variation in virion maturation is due to incomplete cleavage of the viral precursor membrane (prM) protein during the process of exocytosis. Immature prM-containing particles are considered non-infectious as the prM protein caps the receptor binding envelope (E) glycoprotein (Li *et al.,* 2008). The cleavage of PrM to the Pr and M fragments is performed by the Golgi resident protease furin. This process is required for the complete maturation of DENV particles and for the conversion of the viral particles into infectious virions. Rodenhuis-Zybert and co-workers have demonstrated that antibody-mediated entry of immature viral particles facilitates the intra-endosomal cleavage of prM by furin and leads to the conversion of immature-non-infective particles to infectious particles (Rodenhuis-Zybert *et al.,* 2010). This indicates that, in the presence of antibodies, immature DENV particles may contribute to the viral load observed in secondary infections. In **chapter 7**, however, we demonstrated that immature DENV is infectious in immDCs via the receptor molecule DC-SIGN, albeit with a low infectivity. In order to elucidate whether ADE can take place in immDCs, immDCs were infected with immature DENV in the presence of increasing dilutions of dengue- immune serum. We found that, immDCs did not display ADE in the presence of

serial dilutions of homotypic DENV-immune serum, thus as in the case of wild type virus (**evaluated in chapter 6**), immDCs did not contribute to ADE after infection with immature particles.

In **chapter 8**, we evaluated the role of immature dengue virus in disease pathogenesis. Initially, we evaluated the capacity of IgG antibodies recovered from sera of patients with different severity of dengue to react to prM and E proteins. No significant differences were found in the relative binding of immature and wild type DENV particles to the antibodies present in the different severity groups; indicating that the prM response is not contributory to severity. In light of the fact that there were no quantitative differences in the content of anti-prM antibodies between severities of the disease, and that DCs did not exhibit and important participation in ADE, we focused on the quality of the response generated by the sera from different severities in the macrophage cell line P388D1. High concentrations of the polyclonal sera from all the severities of dengue neutralized the infection. Nonetheless, enhancement of the infectivity was observed in the presence of high dilutions of polyclonal sera. In addition, it was observed that ADE of immature and standard DENV in macrophages was influenced by endosomal furin. Taken together, these findings demonstrate that prM antibodies indeed play a role in the entry of immature DENV particles in cells and are consistent with a prior publication of our group demonstrated that antibody-opsonized immature virus requires endosomal furin to become infectious (Rodenhuis-Zybert *et al.,* 2010).

Role of dendritic cells in DENV infection

<u>Dendritic cells vs DENV</u>

Dendritic cells are essential in the detection and response to pathogens, such as viruses. Recognition of the antigens occurs via a set of PRRs including TLRs that activate DC maturation and production of soluble factors including cytokines. These phenotypical and functional changes are required for DCs to become professional antigen presenting cells (APCs). Accordingly, alteration in TLRs signaling can directly affect DC activation, and ultimately lead to aberrant immune responses. We and others have shown that DENV infection can indeed modulate TLR2, TLR3, , TLR4, TLR7 (Modhiran *et al.,* 2010) and TLR9 expression and signaling (Azeredo *et al.,* 2010; Modhiran *et al.,* 2010; Torres *et al.,* 2013; Tsai *et al.,* 2009; Wang *et al.,* 2006) and that this modulation can be associated with different disease presentation. It is thus clear that DCs activation plays an important role in dengue pathogenesis. Importantly, DENV replicates in DCs and although the major targets of DENV infection *in vivo* is still under investigation, DCs are considered to be one of the most important susceptible cell-types. Thus, it seems that DENV infection will be like a battle of 2 giants, one fights the infection and the other exerting mechanisms to block these processes. Since the presence of dengue-specific antibodies during infection may become a sword of each of the two giants, the following part of the discussion is dedicated to the immune responses that might be initiated by DCs during primary and secondary infections.

<u>DCs in primary DENV infections</u>

The typical clinical manifestation of dengue, DF, is characterized by high fever, severe retro-orbital headache, myalgias, and other signs that are thought to result from exaggerated proinflammatory responses. However, epidemiological evidence has demonstrated that a significant number of primary DENV infections are in-apparent or subclinical. Thus, in the majority of primary infections the immune system is able to control the infection. IFN type 1 has been shown to be crucial for the containment of DENV infection (Samuel, 2001). Differential activation of endosomal TLR3, TLR7, TLR8, and TLR9, can thus lead to high or low antiviral responses (Kawai *et al.,* 2004; Okahira *et al.,* 2005) . Indeed, it has been shown *in vitro* that over-expression of TLR3 inhibited DENV replication through IFN production (Tsai *et al.,* 2009). The specific expression of TLRs and likely other PRRs that recognize DENV exhibited by an infected individual may contribute or even predispose to a distinct disease presentation.
In addition, we demonstrated that DENV infection did not induce maturation of DCs or production of fever-inducing cytokines like Il-6 and TNF-alpha, suggesting that DCs were able to abort infection without inducing strong immune responses. This immune-modulatory effect of DENV infection is in line with previous publications (Chase *et al.,* 2011; Jones *et al.,* 2005) and in contrast to others (Ho *et al.,* 2001; Libraty *et al.,* 2001; Nightingale *et al.,* 2008).The lack of unanimity between the studies, although likely reflecting different DENV strains and genetic background of the donors tested *in vitro*, also perhaps mirrors the variety of the disease presentations.

What role will DCs play as DENV factory during primary infection? We showed that immDCs might not be as susceptible to DENV as previously thought. In fact, the majority of immDCs were able to block infection of internalized DENV. Moreover, immDCs that did replicate the virus produced virions having a very low specific infectivity when compared to mosquito cell line- or matDCs-derived viruses. It has been previously reported that in the process of DC maturation, there is up-regulation of N-glycosyltrasferases enzymes. Importantly, during the replication cycle of DENV, these enzymes are responsible for N-glycosylation of Asn-67 and Asn-153 on DENV envelope (E), and both glycosylations are related to higher infectivity of the released viral particles. Whether inefficient glycosylation is the underlining mechanism of the low specific infectivity of immDCs-derived virus remains to be investigated.
In summary, in our studies DENV infection of immDCs did not induce production of pyrogenic cytokines and led to production of poorly infectious virus. Accordingly, during primary infections immDCs are more likely to contribute to the containment of DENV infection rather than to its dissemination. Since our data at least in part corroborated previous studies, we propose that these observations could explain how certain individuals contract the infection without any noticeable signs of disease. Whether this phenomenon does indeed contribute to the resolution of the infection rather than spreading the virus requires further investigation.

On the other hand, matDCs, which produce virus with relatively high specific infectivity, may play important role dengue pathogenesis. Accordingly, it may suggest that primary DENV infection of an individual with ongoing inflammation may confer a risk for more symptomatic or maybe even enhanced infection presentation.

DCs in secondary infections

The vast majority of secondary infections are resolved, signifying that antibodies also confer protection. In this study, we found that in the presence of high concentrations, dengue immune serum neutralized infection in DCs. Under neutralizing conditions, immDCs not only blocked infection but also up-regulated HLA-DR, costimulatory molecules, and secreted inflammatory cytokines suggesting a transition to fully functional APCs. Interestingly, the activation of DCs was found to depend on the ligation of the DENV immune complexes with FcRyIIa and occurred irrespectively of neutralization. A study performed by Den Dunnen and Co-workers revealed that the presence of immune-complexes formed by IgG and *Staphylococcus aureus* triggers maturation and cytokine production by immDCs (den Dunnen *et al.,* 2012). When we evaluated the immunological effects of antibodies during DENV infection of immDCs, we found that immDCs at neutralizing as well as non-neutralizing antibody conditions up-regulated IL-4, IL-6, and TNF-alpha production. At neutralizing conditions, the DENV antigen-positive cells also exhibited a mature phenotype when compared to cells exposed to virus unaided by antibodies. These findings suggest that, during secondary infections, the presence of high concentrations of antibodies not only favors neutralization of the virus but also induces a stronger immune response.

Heterologous secondary DENV infections are mainly interpreted as a risk factor for the development of DHF/DSS. This has been linked to the presence of sub-neutralizing levels of heterotypic antibodies which facilitate ADE and thereby increase the titers of circulating virus. Here we have shown that matDCs likely to contribute to the ADE phenomenon in the presence of sub-neutralizing levels of dengue-specific antibodies. Interestingly, at the same antibody dilution, immDC maturation was affected and a mainly pro-inflammatory response was seen.

High viremic titers often prelude severe disease development. The increased viral burden subsequently triggers an imbalanced immune response which leads to a so called cytokine stormby T lymphocytes (Green & Rothman, 2006; Navarro-Sanchez *et al.,* 2005). Indeed, elevated cytokine levels are seen in patients with DHF/DSS . It has been reported that DENV infection of DCs activates T cells to produce a mixed TH1, TH2 cytokine profile (Chaturvedi *et al.,* 1999). Since TLRs are directly related to cytokine production and maturation of DCs, we sought to find differences in TLR expression profile according to the severity of the disease. Indeed, we found that there is a differential profile in the expression of TLRs in DCs from DHF patients when compared to the DF patients. DCs from severe dengue cases exhibit high expression of the surface-expressed TLR2 and down-regulated expression of the intracellular receptors TLR3 and TLR9. Since TLR2 is known to recognize bacterial products such as peptidoglycans, and lipoarabinomannan its increased expression pDCs from DHF patients is puzzling. It has been reported that the activation of TLR2 is mainly related to the induction of a TH-2 biased cytokine profile, which drives proliferation of the regulatory T lymphocytes (T-regs) (Redecke *et al.,* 2004). This assertion draws us to the conclusion that the activation of TLRs and the induction of a TH2 profile may have two implications regarding the dengue disease. On one hand, a TH2 biased cytokine profile represented mainly by IL-10 may be a regulatory

response to the exacerbated inflammatory environment generated by T lymphocytes during secondary infections. On the other hand, since high levels of IL-10 has been recognized as a severity marker in dengue, it may signify that TH2 skewed cytokine profile contributes to the pathogenesis of dengue by the abrogation of the antiviral response (Redecke *et al.,* 2004). Future studies should elaborate the role of TH2 immunity.

In summary, we propose that there is a direct link between DC activation, antibodies, and T-cell-mediated responses and protection during secondary DENV infections and that this effect is contingent on the concentration of antibodies present during the infection.

Immature DENV particles: Their contribution in disease

DENV-infected cells secrete a heterogeneous population of particles with regard to the number of precursor membrane (prM) molecules per individual particle. Within infected cells, the prM protein acts as a chaperone and blocks the envelope (E) glycoprotein from undergoing pre-mature pH-induced conformational changes during transit through the acidic secretory pathway (Elshuber *et al*., 2003). Indeed, structural studies revealed that the prM protein caps the E protein. Prior to secretion, the prM protein is cleaved into a membrane (M) protein and a pr peptide, the latter dissociates after the release of the particle to the extracellular milieu. This cleavage event is, however, not efficient as almost all particles contain residual prM. Indeed, approximately 90% of mosquito cell-derived DENV appears to contain prM molecules, indicating that during viral transmission many prM-containing immature particles are released into the skin (Junjhon *et al.,* 2010). The functional properties of immature DENV particles and their role in disease pathogenesis has been subject of our study.

Primary infection

We and others showed before that immature DENV particles are non-infectious in cell lines because they fail to bind to receptors expressed at the cell surface (Guirakhoo *et al.,* 1991; Junjhon *et al.,* 2010). We argued that prM caps the receptor binding motif of the E protein thereby rendering them non-infectious. This implied that these particles would not contribute to disease pathogenesis (Elshuber *et al.,* 2003; Moesker *et al.,* 2010; Randolph *et al.,* 1990; Yu *et al.,* 2008; Zybert *et al.,* 2008). Recently, however, another study revealed that immature particles of West Nile Virus (WNV), which is closely related to DENV, is infectious in cells expressing DC-SIGN (Mukherjee *et al.,* 2011). DC-SIGN is abundantly expressed at the cell surface of DCs, cells that are known to be permissive to DENV infection. Therefore, **in chapter 7**, we addressed the question of whether immature DENV particles can use DC-SIGN to enter immDCs. Indeed, we demonstrated that immDCs can be infected with immature DENV particles due to interaction with DC-SIGN. This suggests that immature DENV particles can also contribute to the viral load in patients experiencing a primary infection. I do however think that mature M-containing particles are more important in disease development as the level of infectivity of immature particles was much lower than that of standard virus preparations. Furthermore, in contrast to mature virions, immature particles will be non-infectious in cells lacking or expressing very low levels of DC-SIGN. Indeed, immature particles were found to be non-infectious on murine macrophage cells, which express low levels of DC-SIGN whereas multiplication of standard virus preparations was seen in these cells ((Boonnak *et al.,* 2008). Taken together, although immature particles were found infectious in immDCs, I believe their role in disease pathogenesis during primary infection is rather limited.

Secondary infection

Previous studies performed by our group have revealed that in cell lines, antibodies directed against prM and E can rescue the infectivity of immature DENV2 particles. Infectivity is rescued by: 1) antibody-Fc receptor interaction, and 2) prM to M cleavage upon entry due to the host protease furin. Intriguingly, the infectious properties of immature DENV particles in presence of antibodies are almost as high as standard preparations. This suggests that during secondary infection immature particles can play an important role in disease pathogenesis. **In chapter 7** we aimed to decipher whether immature DENV particles from DENV-1, 3, and 4 also recover their infectivity after opsonization with DENV-antibodies. Indeed, in macrophages, enhanced infectivity of immature DENV1, 2, 3, and 4 was seen in presence of antibodies. In immDCs, however, no enhanced infectivity was observed following immature DENV-2 infection in presence of antibodies. The experiments could not be performed with immature DENV1, 3, or 4 due to too low infectivity of these virus preparations. However, the data obtained with immature DENV-2 is in agreement with our data with standard dengue preparations and suggest that the efficient DENVE/prM-DC-SIGN interaction overrules the additive role of FcR-mediated entry of DENV in the presence of non-neutralizing antibodies. One could also speculate that the intracellular network of PRRs displayed by DCs, such as TLRs, play

an important role in the abrogation of ADE by the activation of antiviral products such as interferon and TH1 profile cytokines. Furthermore, immature particles appear to be less infectious in immDCs than mature virions as we did not -despite the moderate infectivity of immature virions in the absence of antibodies – observe ADE. Also, immature DENV infectivity in the presence of antibodies is less efficient in immDCs than in macrophages. This observation may imply that immDCs do not allow viral maturation upon entry to the cells in the same way as macrophages do. Given the high infectivity of immature particles opsonized with antibodies in macrophages, we wondered whether these particles and the antibodies that recognize them are crucial for severe disease development. As described in **chapter 8**, we found that antibodies from all severities are capable to bind to immature DENV particles without differences. Furthermore, we also corroborate that immDCs are not susceptible to undergo ADE neither in the presence of standard DENV nor immature DENV particles. In macrophages, the immature DENV particles present within the standard virus preparations contributed to the total viral output under ADE conditions but no differences were observed between disease severities. Taken together, antibody-opsonized immature particles are not the sole trigger but act as a co-factor in disease pathogenesis. The heterogeneity of DENV particles thus adds another layer of complexity to dengue pathogenesis.

All neutralizing anti-DENV antibodies analyzed to date caused ADE once the antibody concentration is below the threshold that is required for virus neutralization (Pierson *et al.*, 2007). But which antibodies recognizing immature and mature particles then protect during homotypic re-infection and enhance disease during heterotypic re-infection?.

Interestingly, the most potent neutralizing antibodies are type specific and directed against the domain III of the E glycoprotein. These represent however a minority of the total anti-E response as more than 90% of the antibodies are generated towards the highly conserved fusion loop in domain II. Antibodies against prM have been shown to be weakly neutralizing and are highly cross-reactive to other serotypes. In homotypic re-infection, the high affinity type specific antibodies likely neutralize the newly infecting virus. It is possible however that at the same time prM antibodies and non-neutralizing E antibodies facilitate successful infection of mature and immature particles during homotypic re-infection. But given the presence of the type specific highly neutralizing antibodies the overall balance is oriented towards neutralization of infection. In case of heterotypic re-infections, only the cross-reactive antibodies can bind and these generally have weakly neutralizing properties. The weakly neutralizing properties of E domain II antibodies plus the ability of antibodies to render immature virions infectious therefore sets the stage to antibody-dependent enhancement of infection. Importantly, however, only 2-4% of the individuals experiencing a heterologous re-infection develop severe disease demonstrating that besides antibodies other unknown factors are also involved in disease pathogenesis.

Concluding remarks and future perspectives

In this thesis, we have demonstrated that immDCs play a dual role in DENV infection. They can confer protection against the infection or contribute to the pathogenesis depending on history of previous DENV infection and the maturation state of the DCs upon infection. We demonstrated that immDCs, despite being relatively higher permissive to viral entry than mat DCs, produced viral particles with low infectivity. In addition, we observed that DENV, when unaided by antibodies, did not induce either the maturation of the cells or cytokine production. We therefore infer that during primary infections, the infection of immDCs does not contribute to the high viral output, nor does contribute to the increasing of the levels of cytokines observed in hemorrhagic manifestations. These observations could at least in part explain the fact that high proportion of primary infections are asymptomatic or do not lead the development of a pro-inflammatory response.

Interestingly, infection of immDCs in the presence of neutralizing concentration of antibodies induced maturation of the cells and cytokine production in a FcRyIIa-dependent manner. Immune complexes at non-neutralizing serum titers, however, were still infectious and led to partial DCs maturation and a distinct more pro-inflammatory cytokine response. Consequently, we propose that during homotypic re-infection, high antibody titers induce the induction of pro-inflammatory cytokines and the generation of DCs capable of initiation of adaptive responses. On the other hand, during heterotypic infection, in the presence of non-neutralizing cross-reactive antibodies, immDCs may acquire a phenotype that is impaired in their ability to initiate adaptive immunity. Subsequently, the pro-inflammatory cytokines released by these cells may increase the number of circulating matDCs. These cells support ADE of infection and release highly infectious particles. Therefore, matDCs may contribute to the development of severe dengue. Does the presence of immune complexes that ligate FcyIIa on DCs lead to the development of an

effective antiviral immune response that contributes to disease containment? If so, we could use this knowledge for the development of effective immunotherapies and vaccines.

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